

The role of nuclear FGFR1 and FGF2 in pancreatic cancer

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DECLARATION OF AUTHORSHIP

I declare that the work presented in this thesis is my own, with contributions from others properly cited and acknowledged. The work was performed between July 2010 and October 2013 in the Centre for Tumour Biology, Barts Cancer Institute, Barts and the London school of Medicine and Dentistry.

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ABSTRACT

Patients who are diagnosed with pancreatic ductal adenocarcinoma (PDAC) face a dismal prognosis. One reason for this is the dense stroma that is a characteristic of PDAC, which may preclude drugs from accessing the tumour cells. Pancreatic stellate cells (PSCs) are the key cell responsible for desmoplasia in PDAC and it is becoming clear that they are a promising target for therapy. Over-expression of FGFs and their receptors is a feature of PDAC and correlates with poor prognosis, but whether their expression impacts on PSCs is unclear. The aim of my research was 1) to understand the role and function of nuclear FGFR1 and FGF using 2D based assays; 2) to use a physiologically relevant 3D organotypic model to study the effects of blocking nuclear FGFR1 and FGF2 in PSCs; 3) to assess whether this target could provide a novel therapeutic strategy in PDAC.

At the invasive front of human pancreatic cancer, FGF2 and FGFR1 localised to the nucleus in activated PSCs but not cancer cells. Inhibiting FGFR1 and FGF2 in PSCs, using RNAi or chemical inhibition *in vitro*, resulted in significantly reduced cell proliferation, which was not seen in cancer cells. Cancer cells co-cultured on top of collagen/Matrigel gels together with PSCs showed marked invasion of both cancer cells and PSCs. However, FGFR inhibition blocked invasion of both PSCs and cancer cells. FGFR inhibition resulted in cytoplasmic localisation of FGFR1 and FGF2, in contrast to vehicle-treated conditions where PSCs with nuclear FGFR1 and FGF2 led cancer cells to invade the underlying extra-cellular matrix. Strikingly, abrogation of nuclear FGFR1 and FGF2 in PSCs abolished cancer cell invasion. These findings suggest a novel therapeutic approach, where preventing nuclear FGF/FGFR mediated proliferation and invasion in PSCs leads to disruption of the tumour microenvironment, preventing pancreatic cancer cell invasion. Thus, for

patients with PDAC which is resistant to conventional chemotherapy, targeting the stroma by blocking nuclear FGFR1 and FGF2 in PSCs identifies a novel therapeutic approach.

LIST OF PUBLICATIONS

Stacey J Coleman, Athina-Myrto Chioni, Mohammed Ghallab, Rhys K Anderson, Nicholas R Lemoine, Richard P Grose, Hemant M Kocher. Pancreatic cancer cell invasion is facilitated by nuclear translocation of FGFR1 and FGF2 in stellate cells. Submitted. *EMBO Molecular Medicine*.

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CHAPTER I INTRODUCTION.

1.1 Pancreatic cancer

1.1.1 Epidemiology

Pancreatic cancer has one of the highest mortality rates among malignancies, and is the fifth most common cause of cancer death in the western world^{1, 2}. With a 5-year survival rate of 3% and median survival of less than six months^{3, 4}, diagnosis of pancreatic ductal adenocarcinoma (PDAC) carries one of the bleakest prognoses in all of medicine. Clearly, there is an unmet need for new therapies for this disease. Surgery (pancreaticoduodenectomy) offers the only hope of a possible cure for patients; however, even of those 10% of patients eligible for curative resection, only 21% will survive beyond five years⁵. Furthermore, while treatments may be efficacious in other cancers, PDAC is resistant to cytotoxic agents and radiotherapy, which further impacts on the poor prognosis of these patients.

1.1.2 Environmental risk factors

Epidemiological and genetic studies have shown that pancreatic cancer is a disease of old age², with rare incidence before age 40 and risk of developing the disease increasing 40-fold by 80 years^{6, 7}. Smoking presents a significant risk factor for development of pancreatic cancer, doubling the risk of developing the disease⁸. Obesity, diabetes mellitus, chronic pancreatitis and heavy alcohol drinking (>six alcoholic drinks a day) also pose a possible risk for developing PDAC⁹⁻¹².

1.1.3 Hereditary /genetic risk factors

One of the risk factors for developing pancreatic cancer is a strong familial history of the disease. Individuals with a first degree relative with pancreatic cancer have a 2.3

fold increase of developing the disease . This risk increases as the number of first degree relatives with the disease also increases, suggesting that inheritance of a rare autosomal dominant allele may be responsible for the development of this disease in a familial setting¹³. It has been observed that patients may have chronic pancreatitis for at least 20 years before they develop PDAC. These patients have a severe disease and a high rate of complications. The risk of developing PDAC is even higher with hereditary pancreatitis, with an estimated 70-fold increase in risk¹⁴. Although most of the genetic reasons for clustering of PDAC in families remain unclear, several genes have been identified that increase the risk of PDAC; summarised in Table 1. 1.

Genetic syndrome	Gene (s)	Risk of PDAC (fold)	Reference
Hereditary breast and ovarian cancer syndrome	<i>BRCA1</i>	No effect up to 2.26	^{15 16}
	<i>BRCA2</i>	3.5	¹⁷
	<i>PALB2</i>	Increased	¹⁸
Peutz-Jeghers syndrome	<i>STK11</i>	132	¹⁹
Hereditary pancreatitis	<i>PRSS1/SPINK1</i>	53	²⁰
Ataxia telangiectasia	<i>ATM</i>	Increased	²¹
Familial atypical multiple mole melanoma	<i>CDKN2A</i>	38	²²
Hereditary non-polyposis colorectal cancer syndrome	<i>MLH1, MSH2, MSH6, PMS2</i>	Increased	²⁰

Table 1. 1 Genetic risk factors of PDAC

BRCA1, breast cancer type 1 susceptibility protein; *BRCA2* breast cancer type 2 susceptibility protein; *PALB2*, Partner and localizer of BRCA2; *STK11*, Serine/threonine kinase 11; *PRSS1*, protease, serine, 1 (trypsin 1); *SPINK1*, serine protease inhibitor Kazal-type 1; *ATM*, Ataxia telangiectasia mutated; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MLH1*, MutL homologue 1; *MSH2*, MutS protein homologue 2; *MSH6*, MutS homolog 6; *PMS2*, postmeiotic segregation increased 2.

1.1.4 Current treatment options

The aetiology of PDAC is multifactorial. The predominant patient population is elderly and shows poor health at time of diagnosis. Currently there is no simple reliable detection method for PDAC and the earliest symptoms are non-specific, which means patients present very late with their disease. Currently, complete resection of the pancreatic tumour offers the only hope for long-term cure of PDAC. However, only a minority of patients qualify for surgery²³. This is due to the fact that, at diagnosis, tumours have either involved the major abdominal blood vessels or have metastasised to distant organs²⁴. However, even following surgery, long-term survival is poor and tumours eventually return²⁵. Due to the high incidence of local recurrence, targeted radiotherapy has been suggested; however, long-term benefits of adjuvant radiotherapy have proven inconclusive²⁶. PDAC tumours are hypoxic, which may explain the limited impact of radiotherapy as a treatment option, since radiation therapy relies on the presence of oxygen²⁷.

Currently, the chemotherapeutic agent Gemcitabine (alone) and the targeted small molecule EGFR tyrosine kinase inhibitor Erlotinib (Tarceva, Genentech, in combination with Gemcitabine) remain the only two agents approved for the treatment of PDAC, despite their modest benefits to patient survival²⁴. Gemcitabine was approved after showing clinical benefit compared to 5-Fluorouracil (5-FU) in patients with advanced PDAC, prolonging survival from 4.4 months to 5.6 months²⁸, despite a recent large randomised phase III trial (ESPAC 3) of folinic acid (leucovorin) and 5-FU versus Gemcitabine showed no survival benefit between the two treatments²⁶. The survival benefit of adding Erlotinib to Gemcitabine was even smaller (5.9 to 6.2 months), albeit clinically significant²⁹.

Recently, a four chemotherapeutic drug combination of 5-FU, leucovorin, oxaliplatin and irinotecan (FOLFIRINOX), was administered to patients with metastatic PDAC. This combination chemotherapy approach has shown an increase in median overall survival of 11.1 versus 6.8 months compared with Gemcitabine alone³⁰. Unfortunately, given the high toxicity of this regimen, FOLFIRINOX is suitable only for patients with a good performance status. Secondly, a phase III trial (MPCAT trial) for advanced PDAC, in which the nano-albumin formation of paclitaxel, nab-paclitaxel (Abraxane), administered in combination with Gemcitabine, was demonstrably superior to Gemcitabine alone (median survival 8.5 months vs. 6.7 months, respectively)³¹, with a more favourable toxicity profile than FOLFIRINOX.

One of the possible reasons that most potential and targeted therapies fail in improving the prognosis of patients with PDAC may be in part explained by the diverse influences exerted by the microenvironment on cancer cells, which confound our ability to study the disease *in vitro*. Revealing the underlying molecular mechanisms of the tumour cell-microenvironment cross-talk may help to shed light on the huge discrepancy between relative success and effectiveness of therapies in preclinical assay (predominately 2D cell based assays and xenograft mouse models) and failure in human PDAC. Thus, the focus of my study will be to understand PDAC in the context of its tumour microenvironment.

1.2 Pathophysiology of PDAC

1.2.1 Development of the exocrine pancreas

The pancreas is an endoderm-derived organ and regulates two important physiological functions, digestion of food and glucose (and fat and amino acid) metabolism (Figure 1. 1). In humans, early in embryogenesis, the pancreas

develops from two spatially distinct primordial buds of endoderm (dorsal and ventral) derived foregut tube, which grow, branch, rotate and fuse in response to signals produced by mesodermal tissues including the notochord, aorta and the cardiac mesoderm³².

Golosow and Grostein first showed that growth and morphogenesis of the immature pancreas depended on interactions between epithelial and mesenchymal tissues³³. The molecular basis of these interactions has since been established and, in particular, fibroblast growth factor (FGF) signalling from endothelial cells and mesenchymal cells surrounding the primitive pancreas has been shown to be crucial for pancreatic cell differentiation and maturation³⁴⁻⁴². Furthermore, FGF signalling from the notochord early in embryogenesis is vital for the differentiation of the foregut endoderm towards a pancreatic fate by repressing the expression of sonic hedgehog (Shh) in the region of the endoderm that is destined to become the dorsal pancreatic bud^{39, 43}. This inhibition of Shh signalling induces the expression of pancreatic genes including the homeodomain transcription factor PDX1⁴³⁻⁴⁵. PDX1 is a key determinant of both exocrine and endocrine pancreatic development and, in mice that lack PDX1, pancreas development is arrested at an early stage⁴⁶.

In addition, a number of other important embryonic signalling pathways are involved at various stages of pancreatic development, including FGF, Notch, Wnt, Retinoid and TGF β signalling, which regulate cell differentiation and maturation in a complex but carefully orchestrated process⁴⁷⁻⁵⁴. This information from developmental biology is vital in understanding PDAC pathophysiology, as a number of embryonic signalling cascades are hijacked by the cancer cells for their unrestrained growth.

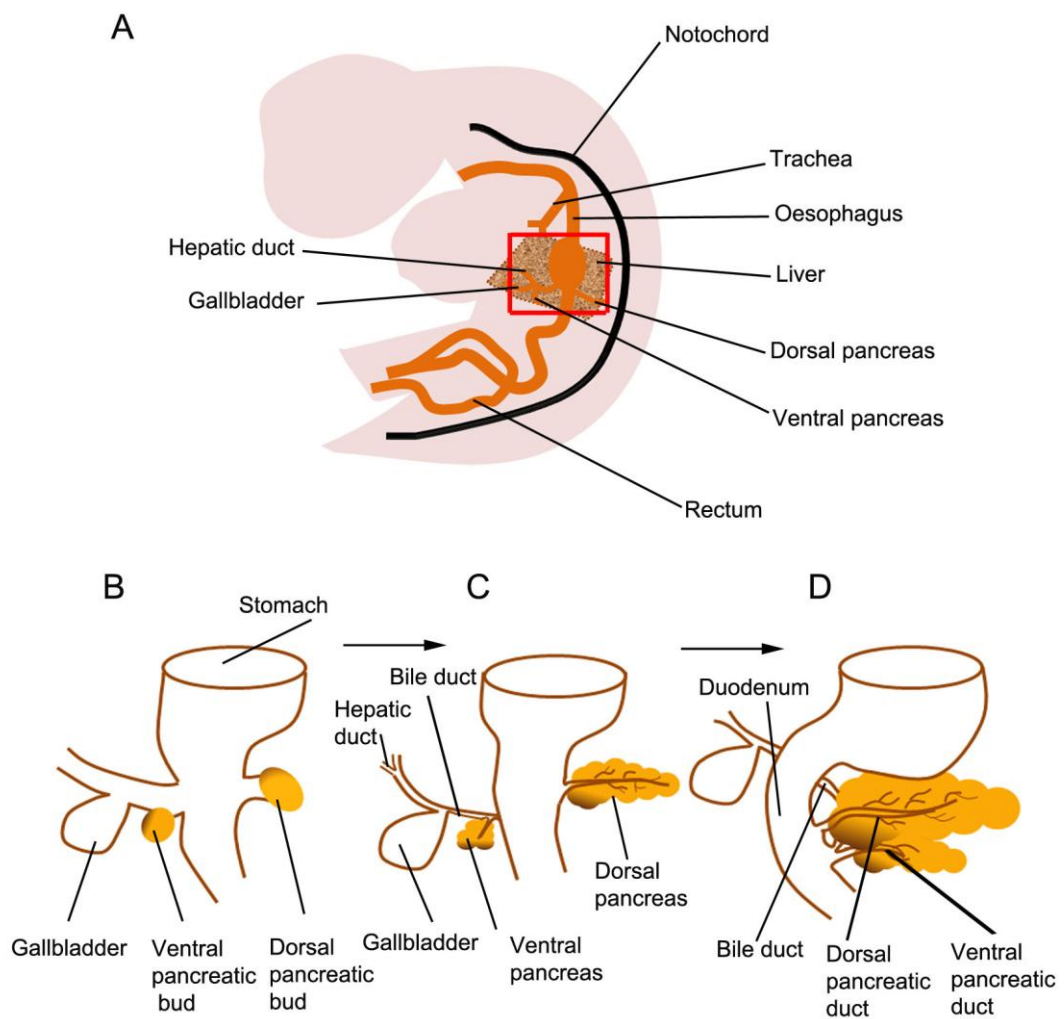


Figure 1. 1 The developing pancreas.

(A) The endodermal development of a 6 week human embryo. The pancreas is represented by two buds that will eventually fuse (shown in red box). (B) Pancreatic development in humans at 30 days, the ventral pancreas bud is close to the liver primordium. (C) By 35 days it begins to migrate (D) and come into contact with the dorsal pancreatic bud.

1.2.2 *Anatomy of the mature pancreas*

The mature pancreas composes morphologically and functionally distinct endocrine and exocrine components. The exocrine portion makes up 95%-99% of the pancreas and consists of acinar and duct cells, which secrete enzymes into the intestine and promote digestion and absorption of carbohydrates, fats, and proteins ducts, forming a dendriform structure that eventually gives rise to the main pancreatic duct (Figure 1. 2)⁵⁵. The small ducts are lined with cuboidal epithelium, while columnar epithelium lines the larger ducts. Acinar cells are pyramidal in shape with basal nuclei, and numerous secretory (zymogen) granules containing the digestive enzymes (proteases, amylases, lipases and nucleases) in the form of inactive precursors. These precursor enzymes become activated when they enter the duodenum. At the junction of the acini and ducts are cuboidal centroacinar cells, which are thought to secrete non-enzymatic components (such as bicarbonate). The endocrine cells are grouped in the islets of Langerhans, discrete cellular aggregates scattered in between the exocrine tissue. The islets consist of insulin producing β cells, glucagon producing α cells, somatostatin producing δ cells and pancreatic polypeptide producing PP cells⁵⁶. Understanding the development, anatomy, histology and physiology of the normal pancreas helps us understand the interactions and cells of origin for PDAC and other pancreatic tumours.

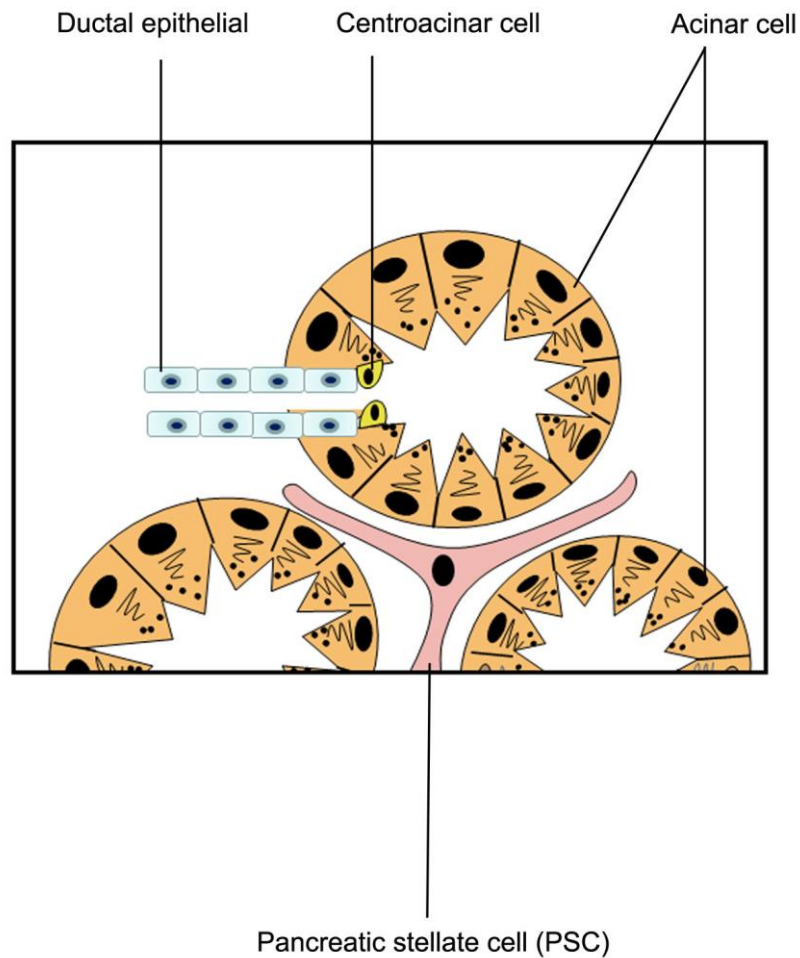


Figure 1. 2 The exocrine pancreas

The pancreatic ductal cell is thought to be the progenitor cell for PDAC; however, studies have shown that other cell types may offer an alternative route to PDAC. Mouse models in which K-Ras is activated specifically in some adult cell types have shown that both acini and insulin positive cells can give rise to PanINs and in some case PDAC. In adult pancreas, exocrine and endocrine components are separate secretory functional units. The exocrine pancreas is composed of acinar units connected to a series of increasingly large ducts that drain into the duodenum. The acinar cell is the major component of the pancreas, making up 90% of its mass. The acinar cells are pyramidal in shape and secrete digestive enzymes into the ducts. In contrast, ductal epithelial cells make up only 10% of the pancreatic mass. These cells are cuboidal in shape and secrete bicarbonate and mucus into the secretory digestive enzyme mixture produced by the acinar cells. In the normal pancreas, quiescent PSCs are present in the periacinar space. These cells have long cytoplasmic processes that encircle the base of the acinus. Centroacinar cells have also been considered as possible multi-lineage pancreatic progenitors^{57 58}

1.2.3 Classification of pancreatic neoplasms

Most cancers of the pancreas are ductal adenocarcinomas and the terms PDAC and PDAC are used synonymously in error⁵⁹. PDAC is characterised by an intense desmoplastic reaction that predominately makes up the cancer mass, with very few neoplastic cells⁶⁰ (Figure 1. 3). Pancreatic cancers are highly infiltrative and vascular and perineural invasion is common, as are metastases to the regional lymph nodes and distant organs. Indeed, most patients present clinically with distant metastasis, most commonly to the liver and peritoneal cavity⁶⁰.

Pancreatic ductal adenocarcinoma is thought, most commonly, to develop through a stepwise accumulation of genetic and epigenetic changes within the pancreatic ductal epithelial cell, despite this representing less than 10% of the pancreatic mass. Broadly, the pre-neoplastic changes are morphologically defined as Pancreatic Intraepithelial Neoplasia (PanIN)⁶¹, representing grades of metaplasia and dysplasia. The lowest grade PanIN lesions are metaplastic flat (1A) or papillary (1B) lesions with absence of nuclear atypia and retained nuclear polarity. PanIN 2 dysplastic lesions show evidence of nuclear atypia and infrequent mitosis, while PanIN 3 lesions have characteristics of carcinoma *in-situ*, demonstrating widespread loss of polarity, nuclear atypia and frequent mitosis (Figure 1. 4)⁶².

1.2.4 Molecular genetics of PDAC.

PDAC is a disease of somatic mutations, with a number of signature genetic alterations that define the disease, with a very few known germline mutations playing a role. Of these frequently observed somatic alterations, activating point mutations at codon 12 of the Kirsten Ras (*K-RAS*) gene are among the most

common genetic alterations⁶³, although occasionally mutations are found in codons 13 or 61. Mutations in *K-RAS* are found in nearly 100% of advanced pancreatic tumours and early PanIN lesions, suggesting that aberrant activation of this protein may be one of the earliest events in the malignant progression of PDAC⁶³. *In vitro* abolition of K-RAS alone can arrest cancer cell growth⁶⁴. Despite this, trials using pharmacological inhibitors that target K-RAS, by inhibiting post-translational modification, have shown no clinical efficacy⁶⁵. K-RAS mutations are also found in patients with chronic pancreatitis who do not develop PDAC⁶⁶ suggesting that K-RAS mutations may be the initial driving force in PanIN development but, alone, are not sufficient to induce PDAC. Indeed, *in vivo* studies in which wild type K-RAS was overexpressed in mice showed this to be sufficient to induce chronic pancreatitis and cause the development of PanIN lesions. However, when combined with the heterozygous loss of *p53* tumour suppressor gene the lesions progressed to PDAC.⁶⁷ Inactivation of tumour suppressor genes *p53*, *SMAD4* (*DPC*) and *BRCA2* are thought to occur later in pancreatic neoplastic progression^{68, 69}. P53 serves as a DNA checkpoint regulator in response to mutations from reactive oxygen species and also telomere shortening, both of which are seen in pancreatic cancer. SMAD4 is a part of the TGF β receptor signalling pathway, relaying signals from the cytoplasm to the nucleus, and loss of SMAD4 confers a growth advantage to cells.⁷⁰ BRCA2 is important for double strand break repair and, thus, important for genomic stability⁷¹. Loss of *BRCA2* leads to a number of chromosomal aberrations in PDAC. Both *P53* and *SMAD4* are inactivated in over 50% of late stage PDAC, whereas *BRCA2* is inactivated in 7-10% pancreatic carcinomas⁶². Finally, studies using a panel of microsatellite markers have shown that all high grade tumours exhibit loss of heterozygosity at more than one locus on chromosomes 3-6,8-11,13,16-18, underscoring the complex genetic composition of PDAC at this late stage⁷². Of note,

none of these have been effectively targeted due to a multitude of factors; one of which is the tumour-stroma cross-talk mediated by a number of signalling cascades.

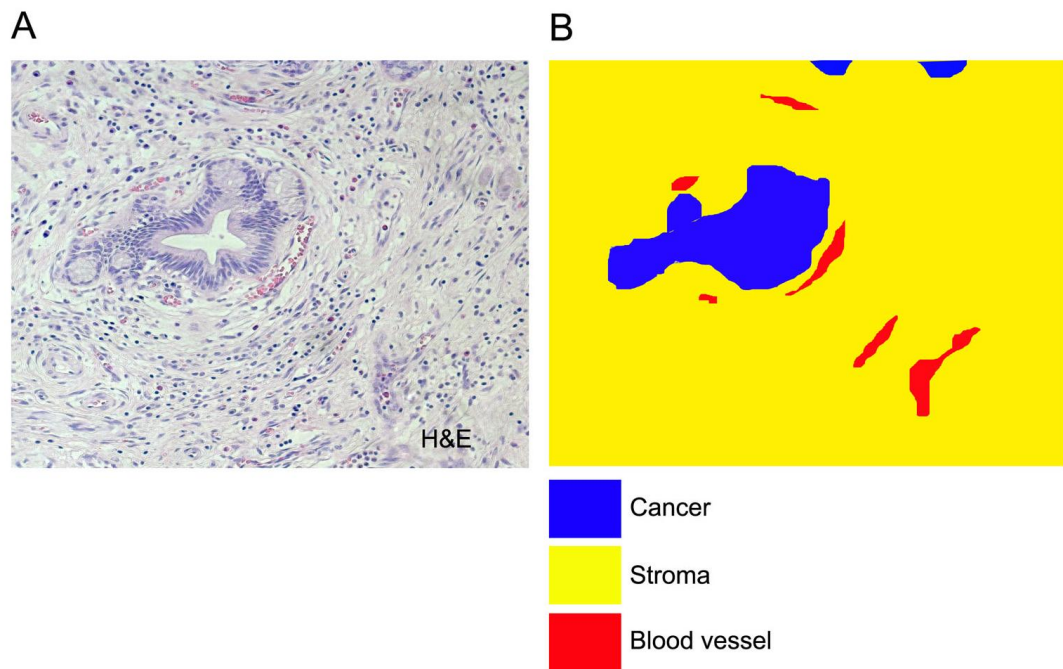


Figure 1. 3 Histology of PDAC

Haematoxylin and eosin (H&E) staining of PDAC (A) and its schematic representation (B), demonstrating the extensive desmoplastic stroma in PDAC.

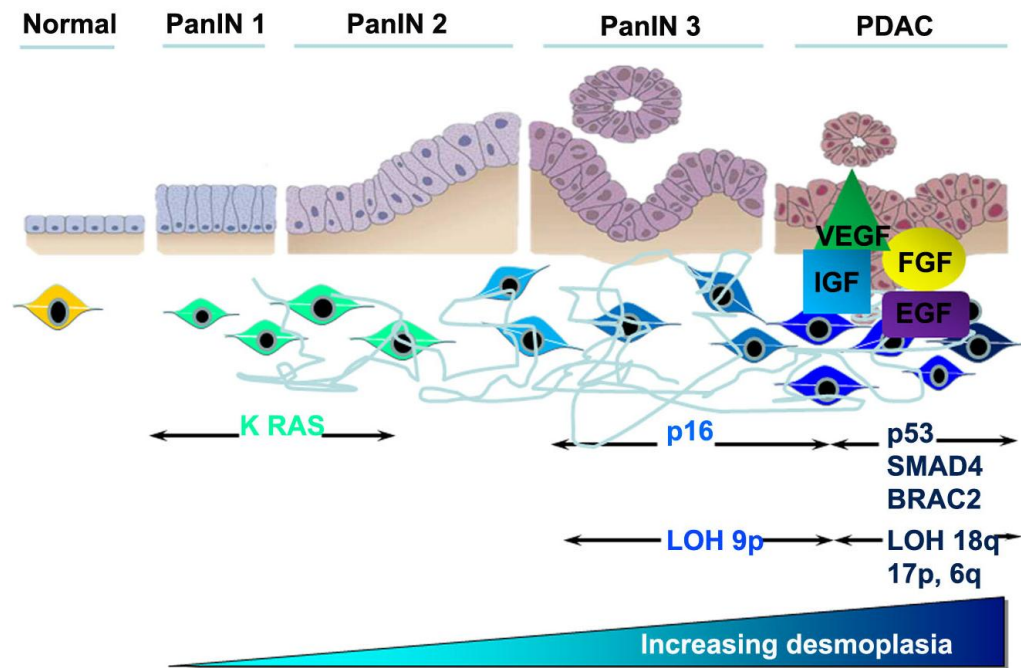


Figure 1. 4 Stepwise development of PDAC

PanINs seem to represent progressive stages of neoplastic growth that are precursors to PDAC. As the adenocarcinoma progresses from low grade PanIN 1A to high grade PanIN 3 the tumour will acquire genetic mutations that seem to occur in a stepwise fashion. Increasing desmoplastic reaction accompanies the progression of low grade PanIN to PanIN3 tumour, a characteristic of PDAC. As well as histological and genetic alterations, PDACs frequently show over expression of a number of growth factors and cytokines.

1.2.4.1 Activation of developmental signalling cascades in PDAC

Ultimately, cell differentiation and pancreas organogenesis is achieved by a subtle balance and well orchestrated pattern of gene expression and interaction of signalling pathways. Studies using genetically engineered mouse models have shown that many critical embryonic signal transduction pathways, quiescent in the adult pancreas, are re-activated during PDAC progression. Important pancreatic developmental signalling pathways such as Notch⁴⁹, TGF β ⁷³, EGF⁷⁴, Wnt⁷⁵, Hedgehog⁷⁶ and FGF⁷⁷, are all deregulated in pancreatic cancer. As the role of FGF signalling in PDAC is the focus of my studies, this signalling pathway will be discussed in detail in section Chapter IV.

In vivo studies have shown that Shh signalling acts on the stromal (mesenchymal) compartment of PanINs and PDAC and can activate the characteristic desmoplastic reaction seen in PDAC⁷⁸. Hh signalling occurs when the Hh ligands (Sonic Hedgehog, Indian Hedgehog or Desert Hedgehog in mammals) are secreted and bind to the Patched receptor (Ptch) on neighbouring cells. This ligand- receptor interaction results in Ptch inhibition and consequential release of the G protein coupled receptor Smoothed (Smo), leading to a cascade of events and the translocation of the Gli transcription factor to the nucleus⁷⁹. As discussed earlier, suppression of Hh signalling is critical for normal pancreas development. However, in PDAC it appears sustained activation of this pathway enhances tumour growth, as shown in human tumour samples and confirmed in a genetically engineered 'KPC' mouse model of PDAC^{76, 80}. Upon treatment of mice, engineered to develop PDAC, with a small molecule inhibitor of Smo, the stromal architecture of their PDAC reorganised and the desmoplasia resolved, increasing tumour vascularity and

facilitating efficient delivery of the chemotherapeutic agent Gemcitabine. As a result, metastasis decreased and the overall survival of mice treated with the combination of Smo inhibitor plus Gemcitabine was significantly increased compared to control⁸¹. These studies suggest that targeting the stromal compartment of PDAC may improve delivery and efficacy of chemotherapy. In light of these results several clinical trials have been initiated to investigate the effects of treatment with Shh inhibitors in patients with PDAC. Unfortunately, disappointing Phase II clinical trial results, with the Smo inhibitor IPI-926 (Saridegib, Infinity Pharmaceuticals) have emphasised the need to fully understand this tumour micro-environment²⁴.

1.3 Pancreatic stroma

The role of the microenvironment in tumour metastasis was first described in 1889 by the English surgeon Stephen Paget as the so-called 'seed and soil' hypothesis. In this hypothesis, Paget described how metastasis was not a random event, but that certain tumour cells ('seed') have a specific affinity for a specific organ environment ('soil'). Only when the 'seed' and 'soil' were compatible, did the metastasis occur⁸². Over a century since this original hypothesis, it is now well appreciated that, in addition to its importance at distant metastases, the 'soil', or tumour microenvironment, at the primary tumour site also plays an important role in the development of the tumour.

Indeed, many epithelial malignancies, including breast, prostate and PDACs, often exhibit a significant stromal reaction around the tumour cells⁸³⁻⁸⁶. The stroma not only functions as a mechanical barrier but also constitutes a dynamic compartment that is critically involved in the process of tumour formation, progression, invasion, and metastasis. In particular, PDAC shows the most prominent stromal reaction or 'desmoplasia' (defined as proliferation of fibrotic tissue with an altered ECM which

contributes to tumour growth and metastasis) and destruction of the normal tissue architecture of all epithelial tumours⁸⁷. The desmoplastic tumour environment is an highly heterogeneous and complex mixture of abundant ECM proteins, growth factors, cytokines and cells from different lineages such as fibroblasts, pancreatic stellate cells, smooth muscle cells, immune and inflammatory cells, endothelial cells⁸⁸. The desmoplasia in PDAC is associated with an increased and abnormal expression of type I collagen, which facilitates the malignant phenotype of tumour cells by promoting increased proliferation and invasion of cancer cells, via activation of $\alpha 2\beta 1$ integrin, and also promotes drug resistance⁸⁹. Collagen I expression in PDAC can also increase the expression of snail and modulate E-cadherin, both of which are important in EMT and increase invasiveness of cancer cells^{90, 91}.

1.3.1 Tumour stroma interactions

The high proportion of stromal cells in PDAC (epithelial cells frequently account for less than 20% of the tumour volume in PDAC⁹²) is associated with over expression of growth factors such as vascular endothelial growth factor (VEGF), EGF, TGF β , insulin-like growth factor I (IGF-I), FGF and their respective receptors, as well as secretion of matrix metalloproteinases (MMPs), which serve to fuel pancreatic cancer growth, metastasis and invasion⁹³. In turn, PDAC cells secrete growth factors such as FGFs, TGF β , IGF and PDGF, which are sequestered in the activated stroma⁵⁸. Invading cancer cells produce MMPs that are able to release these stored growth factors. MMPs are zinc-dependent proteinases that are frequently overexpressed in cancer⁹⁴. Data suggest that activation of MMP-2 and MMP-9 plays a key role in cancer invasion and metastasis, by digesting the ECM and aiding in the desmoplastic reaction in PDAC⁹⁵. Cancer cells also shed glypican 1 and syndecan 1, heparan sulfate proteoglycans which can modulate paracrine growth factor

signalling^{96, 97}. In addition to the changes observed in cancer cells, this interaction between cancer cells and stroma leads to altered transcription in stromal components such as fibroblasts and inflammatory cells, promoting cancer cell motility (via altered integrin expression patterns) and resistance to hypoxia (via altered expression of VEGF A and hypoxia inducible factor 1 α , HIF1 α)⁹⁸. The net result is a unique micro-environment consisting of aberrant epithelial and mesenchymal cells, in which pancreatic cancer cells thrive⁹². Whilst it is clear that the tumour-stroma interactions are critical in development, progression and maintenance of PDAC, model systems to study these experimentally have only been available in the last decade. Briefly there are two distinct groups: *in vivo* and *in vitro* model systems.

1.3.2 *In vivo* model systems

The development of genetically engineered mouse (GEM) models of PDAC has provided the most physiologically relevant model that closely mimics the situation in human cancer. Most of the GEM models of PDAC are based on the conditional, pancreas-specific, expression of the *Kras* oncogene (*KRAS*^{G12D}), present in 90% of human PDAC cases⁶³. This is facilitated by expressing Cre recombinase under the control of the promoter of the embryonic pancreas lineage determining transcription factor Pdx-1 or Ptf1/p48 gene ('KC' mice). KC mice develop pancreatic tumours ranging from precursor PanINs to fully invasive and metastatic disease^{99, 100}, albeit with a long latency period of up to a year. These KC mice have been crossed with mice harbouring several additional mutations, to investigate their contribution to the rapid progression to PDAC. GEM models of PDAC have been developed with activating mutations in TGF β receptor and/or inactivation of tumoral suppressors

such as p53 ('KPC' mice), INK4A/ARF and Smad4, which are the most common PDAC drivers¹⁰¹. A brief summary of the various GEM available is shown in Table 1. 2 and there are several excellent reviews on the various GEM models that have been developed for studying the development of PDAC¹⁰²⁻¹⁰⁴. The generation of complex allele combinations, together with the latency period involved in the development of tumours, makes these models inherently expensive. Further criticism against GEM models of PDAC has focused on the multi-focality of their PDAC, involvement of whole pancreas with tumours, histological variants commonly observed, presence of tumours in other organs and genetic homogeneity; features not associated with human PDAC. In addition, the interplay of various signalling interactions from the diverse cell types present in the tumour makes the contribution from key epithelial-stromal interactions in PDAC impossible to study. The use of organotypic cultures using pancreatic stellate cells (PSC), the key cells driving the desmoplastic reaction, has been made feasible only recently¹⁰⁵.

Gene/promoter	Phenotype of mouse	References
Transgenics with predominantly acinar phenotypes		
T-Ag/Ela	Acinar cell carcinoma	106
Hras/ Ela	Acinar cell carcinoma	107
TGF α / Ela	Acinar cell carcinoma	108
c-myc/ Ela	Mixed acinar-ductal tumours	109
KRAS ^{G12D} /Mist1	Acinar cell carcinoma	110
Activated KRAS knock in GEMs		
KRAS ^{G12D} PDX1-Cre	Spectrum of PanINs some mice develop PDAC after long latency	111
KRAS ^{G12D} PDX1-Cre Ink4a ^{-/-}	Develop PDAC with shorter latency than KRAS mutant alone	112
KRAS ^{G12D} PDX1-Cre Ink4a/Arf ^{-/-}	Develop PDAC with high penetrance and short latency. Micrometastatic disease	112
KRAS ^{G12D} P48-Cre Tgfbr2 ^{-/-}	Accelerated PanIN and PDAC development	113
KRAS ^{G12D} PDX1-Cre p53 ^{R273H}	Develop PDAC with high penetrance. Metastasis	114
KRAS ^{G12D} PDX1-Cre Ink4a/Arf ^{+/-}	Develop PDAC with longer latency than Ink/Arf null mice. Metastasis	115
KRAS ^{G12D} PDX1-Cre p53 ^{+/-} Ink4a/Arf ^{+/-}	Develop PDAC with high penetrance and shorter latency than p53 ^{-/-}	115

Table 1. 2 Mouse models of PDAC.

T-Ag, SV40 large T antigen; H-RAS, Harvey RAS; Ela, elastase promoter; Mist1 a transcription factor important in acinar cell maturation .The expression of Mist1 is restricted to acinar cells and is not expressed in duct cells or fibroblasts within the pancreas¹¹⁶.

1.3.3 Pancreatic Stellate Cells (PSCs)

1.3.3.1 Isolation and characterisation of PSCs

Hepatic stellate cells (HSCs) were first described by Karl van Kuppfer in the 19th century as '*sternzellen*', which were able to store vitamin A (retinol) as droplets in their cytoplasm¹¹⁷. A striking characteristic of stellate cells is the capability of these retinoid droplets to exhibit a rapidly fading blue green autofluorescence when excited with light at ~328nm¹¹⁸. Since the identification of HSCs, retinoid storing stellate cells have also been identified in other organs such as the kidney, intestine, spleen and lung¹¹⁹. However, it was only as recently as the 1980s, that the pancreatic stellate cell (PSC) was first characterised. Similar to HSCs, PSCs are able to store retinol in their cytoplasm and, subsequently, PSCs were identified, using autofluorescence of retinoids and electron microscopy, in mice that had been fed vitamin A^{58, 120}. These cells have also been identified in normal rat and human pancreatic tissue. Studies later showed that, in patients with chronic alcoholic pancreatitis, lipid storing cells were abundant in the areas of fibrosis and were capable of secreting extracellular matrix (ECM) proteins^{121, 122}. These studies suggest PSCs were a possible source of pancreatic fibrosis, similar to that has been observed in alcohol related liver fibrosis.

In health, PSCs exist in a quiescent state¹²³ and are a normal resident cell in the exocrine pancreas, where they are thought to make up 4-7% of the pancreatic cell types¹²⁴. PSCs are present in the periacinar space and are characterised by long cytoplasmic processes that encircle the base of the acinus. They may also be present in the perivascular and periductal regions of the pancreas. PSCs express the marker proteins desmin and glial fibrillary acidic protein (GFAP, absent in fibroblasts), together with the presence of intracellular lipid droplets, which serve to

distinguish these cells from pancreatic fibroblasts¹²⁵, present in the interlobular septum.

Given that PSCs express many proteins that are characteristic of several cell types (desmin expression in monocytes, GFAP in astrocytes, nestin in neuroepithelial stem cells and vimentin in mesenchymal cells), the origin of stellate cells is still under debate. Most of the understanding of the origin of stellate cells has emerged from studies of HSCs, in which mesenchymal, endodermal and neuroectodermal origins have been suggested¹²⁶⁻¹²⁸. Recently, studies using *Wilms Tumour 1* promoter (*Wt1*)^{CreERT2} / Rosa26LacZ^{flox} mice have shown that HSCs can arise from a mesodermal origin¹²⁹. Given that gene expression and functional studies have recently shown that HSCs and PSCs share many homologies (expression of genes related to ECM proteins, contractility, retinoid metabolism and expression of growth factors), but are distinctly different from normal fibroblasts^{126, 130}, it is likely that PSCs and HSCs may share a common origin.

The isolation and immortalisation of PSCs from human and rat pancreas has provided an additional tool for studies of the effect of PSC activation. PSCs have been immortalised by SV40 large T antigen in rat PSCs or by SV40 large T antigen and human telomerase in human PSCs¹³¹. The resultant immortalised PSC line is comparable to activated PSCs, showing expression of SMA and ECM proteins. Importantly, DNA microarrays used to compare primary and PSC cell lines have shown only a few differences, including differences in expression of ECM proteins, cytokines and integrins¹³². In addition, both immortalised and primary PSCs respond to TGF- β and PDGF in a similar manner¹³³. The use of primary and immortalised

PSCs, as well as co-culture systems, has given us a mechanistic insight into the biology of PSCs.

1.3.3.2 Activation of PSCs

During injury, PSCs have the ability to trans-differentiate from a quiescent lipid storing phenotype to a myofibroblastic-like activated state (with loss of lipid droplet storage), producing α -smooth muscle actin (α SMA) and secreting excessive amounts of the ECM proteins, such as collagen I, that comprise fibrotic tissue (Table 1. 3). Activation of PSCs is also associated with several morphological changes, including nuclear enlargement and enhanced prominence of the endoplasmic reticulum network, suggesting an increase in protein synthesis⁵⁸.

Recent studies have shown that a small proportion of activated PSCs may also be derived from circulating bone marrow (BM) derived cells that are attracted to the pancreas during injury¹³⁴. In this study, irradiated female mice were injected with GFP expressing pluripotent BM cells isolated from male mice, followed by the induction of chronic pancreatitis with repeated injections of cerulein. The results consistently showed an induction of chronic pancreatitis, with an increase in GFP positive PSCs. However, the contribution of GFP positive PSCs accounted for less than 20% of PSCs found within the diseased pancreas. Therefore, it is likely that the majority of PSCs seen in response to injury are pancreatic derived. It is unknown if BM derived cells may be responsible for a sub-population of PSCs in human fibrosis.

PSCs are activated when primary cells are cultured on plastic and also as a result of injury, including oxidative stress, toxins such as alcohol and exposure to growth

factors¹³⁵. *In vitro* culture of rodent and human PSCs has highlighted numerous growth factors, cytokines, hormones, intracellular signalling molecules and transcription factors that regulate activation of PSCs. It is possible that *in vivo* paracrine factors such as cytokines (IL-1, IL-6, IL-8 and TNF α), growth factors (PDGF, TGF β and FGFs), angiotensin II, and reactive oxygen species from damaged neighbouring cells and leukocytes can lead to PSC activation^{105, 136-141}. Activated PSCs can, in turn, secrete growth factors (such as PDGF, TGF β), cytokines (IL-1, IL-6 and TRAIL) and pro-inflammatory molecules (COX-2), which can all serve to fuel the activation state of PSCs in an autocrine manner^{138, 140, 142, 143}. A number of key signalling pathways are able to mediate regulation of PSC state. The MAPK pathway serves to transduce signals from growth factors as well as stimulation by ethanol. PI3K, RHO kinase, JAK/STAT, NF- κ B and the TGF β /SMAD pathways can also regulate PSC activation¹⁴⁴⁻¹⁴⁶.

Parenchymal necrosis and inflammation is thought to be a requisite for activation of PSCs. This has been confirmed using time-lapse studies with animal models, which have indeed shown that inflammation precedes activation^{147, 148}. Once activated, PSCs show increased proliferation and migration as well as synthesis of ECM proteins (including collagen I and fibronectin¹⁴⁹), leading to fibrosis or ECM remodelling as part of the response to injury. PSCs play a key role in the extensive tissue fibrosis that accompanies chronic pancreatitis, leading to destruction of the normal exocrine function¹⁵⁰. Although the exact role of PSCs in the repair processes following injury remains to be elucidated, it is thought PSCs present in the pancreas probably contribute to the formation of a matrix that allows proliferation, migration and generation of new pancreatic cells¹⁴⁷.

By secretion of ECM proteins and proteases such as MMPs and their inhibitors, PSCs can modulate the surrounding matrix¹⁵¹. In most studies in which PSCs are activated after damage to the pancreas, the inflammation processes resolves and activated PSCs eventually disappear after the cessation of the inflammation mediator⁵⁸. However, repeated inflammatory damage and the failure of the mechanisms regulating tissue repair can lead to chronic inflammation, persistent PSC activation and proliferation, eventually giving rise to fibrosis. Indeed, continuous episodes of experimental pancreatitis produce changes that resemble chronic pancreatitis^{152 151}. Furthermore, changes in the composition of the ECM during repair processes can modulate PSC activation. For example, activated PSCs can be reverted back to a quiescent state when cultured on basal membrane like matrix suggesting ECM composition can modulate PSC behaviour¹³³. Fibrosis in the pancreas and other organs is very much a wound healing response to repeated damage such as chronic alcohol consumption, pancreatic duct obstruction, metabolic disorders, and genetic defects. This chronic injury only serves to exaggerate the activated PSC phenotype, thus fuelling and sustaining fibrosis¹⁵³. Unsurprisingly then, activated PSCs are now thought to be the source of fibrosis in PDAC.

	Quiescent	Activated
Lipid droplets in cytoplasm	+	Absent
Endoplasmic reticulum	+	++
Nucleus	Basal size	Enlarged
Vimentin expression	++	++
Desmin expression	+	+
GFAP expression	+	+
Nestin expression	+	++
α -SMA	-	+
ECM production	Limited	Increased
Cell proliferation	Limited	Increased
Cell migration	Limited	Increased

Table 1. 3 Features of quiescent and activated PSCs

- Absent; +presence; ++ abundant⁵⁸

1.3.3.3 Role of activated PSCs in PDAC

The link between PSCs as a source of desmoplasia and PDAC was identified relatively recently. PSCs were shown to be the specific cellular source of ECM proteins and the predominant source of collagen in the tumour stroma of PDAC patients¹²¹. More recent studies have shown that activated PSCs are also present in early PanIN lesions both in human PDAC as well as tumours that arise in the KPC mouse model, suggesting they may play a role in early PDAC development¹⁵⁴.

1.3.3.4 Evidence of interaction of activated PSCs and pancreatic cancers cells (PCCs) from in vitro studies

In vitro studies have shown that pancreatic cancer cells (PCCs) and PSCs can exist in a symbiotic relationship (Figure 1. 5) PCCs stimulate proliferation and migratory potential of PSCs and lead to an increased production of ECM proteins¹²². PCCs secrete a number of potent mitogens such as TGF- β 1, FGF, PDGF and VEGF¹⁵⁵, all known to activate PSCs. It is likely that TGF β and FGF2¹²² are able to mediate activation of PSCs to induce ECM production (this phenotype is inhibited by neutralising antibodies to both TGF β 1 and FGF2), whereas proliferation of PSCs is promoted by PDGF¹⁵⁵. PCCs are also capable of secreting extracellular matrix metalloproteinase inducer (EMMPRIN), which leads to the increased secretion of matrix metalloproteinase 2 (MMP2) by PSCs, a factor which can induce PCC invasion¹⁵⁶. Furthermore early PanIN lesions that are isolated from KPC mice are capable of inducing activation and proliferation of PSCs, suggesting that even early pre-neoplastic lesions can activate PSCs¹⁵⁷.

In turn, PSCs are able to stimulate cancer cell proliferation and at the same time inhibit apoptosis, fuelling an increase in tumour cell population. PSCs also increase the migration of PCCs and induce epithelial to mesenchymal transition, as shown by the increased expression of the mesenchymal markers snail and vimentin, and reduction in the epithelial marker E-cadherin⁵⁸, by yet to be determined secretory or other mechanisms.

Moreover, the culture supernatant of PSCs isolated from PDAC has been shown to significantly promote proliferation and colony formation of human pancreatic duct epithelial cells (HPDE). Many genes involved tumourigenesis were upregulated in HPDE cells incubated with PSC conditioned media; including *FGF 2*, *PTEN*, *IL13RA2*, *ANXA2*, *CXCL2*, *KLF6*, *IL8*, *SOX8*, *CCR3*, *SP5* and *MYCN*, while tumour suppressor genes were down regulated (including *CASP14*, *FN1*, *TEP1*, *BMP7*, *CX3CL1* and *WNT10A*)¹⁵⁸. These results suggest PSCs, through paracrine factors, can promote the transformation of normal duct epithelium into malignant cells.

The desmoplasia in PDAC is believed to have a detrimental effect on the successful response to chemotherapy and radiotherapy^{81, 156}. PSCs have been shown to protect PCCs from radio- and chemotherapy by increasing PCC clonal survival in a β 1 integrin dependent manner¹⁵⁹. PSCs are also able to increase the 'stem cell' phenotype of PCCs (shown by induced expression of cancer stem cell related genes *ABCG2*, a membrane transporter protein which is highly expressed in cancer stem cells,¹⁶⁰ *Nestin*, a marker of pancreatic progenitor cells¹⁶¹ and *LIN28* which maintains embryonic stem cell functions¹⁶²), a possible mechanism of resistance to therapy¹⁶³. Therefore, PDAC cells and PSCs together establish a vicious cycle of mutually reinforcing mechanisms to sustain the desmoplastic reaction.

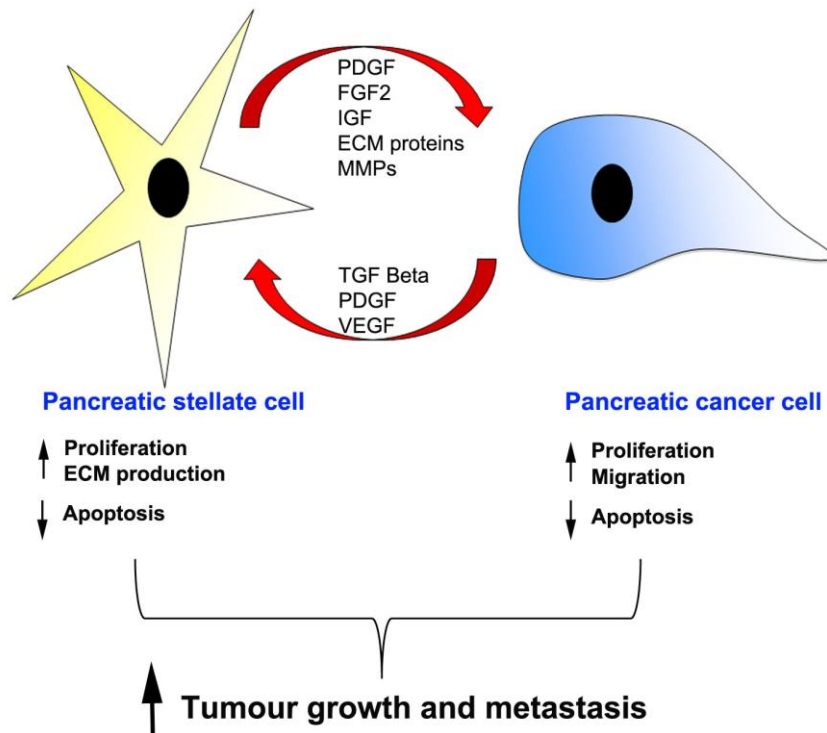


Figure 1. 5 Interaction of PSCs and PCCs

There is accumulating evidence for PSC cross talk with pancreatic cancer cells, which in turn contributes to tumour desmoplasia in PDAC. Such cross talk can promote tumour growth and metastasis^{121, 156, 164},

1.4 Organotypic culture models of pancreatic cancer

Many of the *in vitro* studies that have demonstrated the interaction of PSCs and PCCs have been carried out in 2D cultures, which do not give an optimal representation of the *in vivo* situation. Cells grown on 2D tissue culture plates or in Transwell inserts differ in their morphology, differentiation and cell-cell and cell-matrix interactions compared to cells *in vivo*. For studying cell behaviour in an *in vitro* model that is easily amenable to experimental manipulation, but more relevant to the physiological environment of PDAC, 3D culture systems can be used¹⁶⁵.

Pancreatic cancer cell lines and normal HPDE cells previously have been cultured on type I glycosaminoglycan scaffolds and in Collagen type I or Matrigel. Given only pancreatic cancer cell lines alone were used in these models, the effect of the stroma on tumour cell behaviour was absent¹⁶⁶⁻¹⁶⁸. However, these studies were able to show that pancreatic cancer cells embedded into Matrigel formed spheroids with a distinct morphology and loss of apico-basal polarity compared to culturing in 2D¹⁶⁶.

The introduction of stromal cells in PDAC 3D organotypic cultures was first demonstrated by our laboratory¹⁶⁵. In these studies, activated PSCs were used to better simulate the tumour microenvironment seen in PDAC. Depending on the question that is being asked, 3D models of PDAC can be set up in various ways using PSCs and PCCs. In order to understand the influence of PCCs on the behaviour of PSCs that have invaded into the stroma, these cells can be embedded in an ECM gel composed of collagen and Matrigel. PCCs embedded into an ECM gel form spheroids that recapitulate the features of glandular epithelial cells *in vivo*¹⁶⁵

Submerging the ECM gels, (with PCCs grown on top of the gel in which the PSCs are embedded) allows us to understand the early events in tumour progression. When PCCs are cultured on top of this model, they form luminal structures that resemble ducts *in vivo*. Using this model, our group has shown that PSCs induce changes in PCC cell adhesion molecules such as Ezrin and that Ezrin translocation from the apical to the basal compartment of the cells is an early event in PCC invasion¹⁶⁹. Finally, in order to study the invasion of PCCs in the 3D model, the submerged culture system can be raised upon a grid ('air-liquid' model) and fed from underneath, creating a gradient that stimulates PCCs to invade. Using the air liquid 3D model, our group has shown that the presence of PSCs leads to a significant increase in, and altered cellular distribution of, β -catenin in PCCs. Interestingly, treating these 3D co-cultures with all trans retinoic acid (ATRA, which renders PSC quiescent) results in a decrease in Wnt signalling and, thereby, reduced PCC cell invasion¹⁷⁰.

Furthermore, 3D organotypics provide a perfect model to isolate cancer cells grown in these cultures and assess changes in signalling cascades and molecular targets due to cancer cell desmoplastic cross talk, which would not be apparent if cancer cells were grown on a 2D platform. In addition, using a 3D culture model reduces the noise from other stromal elements present *in vivo* and focuses on the cancer-stromal interaction. Using this approach, we have previously isolated pancreatic cancer cells from organotypic models in which PSCs were embedded in the ECM gel and used gene expression microarrays to demonstrate that cancer cell stromal interactions significantly alter proliferation, cell cycle, cell movement, cell-cell signalling and inflammatory response in addition to changing stiffness in the ECM gel¹⁷¹. Importantly, this study also showed that changes in stiffness of ECM gels was

particularly prominent as the proportion of PSCs in the ECM gel increases, which is highly pertinent to drug delivery and perfusion in PDAC⁸¹. This study also highlights the possible value of multidrug targeting in PDAC in order for chemotherapy or radiotherapy to be effective, as many of these cancer cell pathways influenced by the stroma are redundant.

Upregulation of Src activity has been shown to correlate with poor prognosis in PDAC and is a possible therapeutic target¹⁷². Despite preclinical studies showing promise of Src inhibitors in combination with chemotherapy, treatment only reduced metastasis in KPC mice by 50%, which may be explained by a failure of drug treatment reaching the tumour¹⁷². The accumulation of ECM component in PDAC distorts the normal architecture of the pancreas tissues, compressing blood vessels leading to reduced perfusion and delivery of therapies to tumour cells. As such, several groups are researching into modulating the stroma in PDAC and examining its potential impact on tumour perfusion. Focusing on Src kinase activity, Anderson and colleagues (using fluorescence lifetime imaging microscopy (FILM) to measure fluorescence resonance energy transfer (FRET) of PDAC cells expressing an ECFP-YFP Src reporter) have recently exploited the use of PDAC organotypic cultures in conjunction with *in vivo* models to investigate the tumour environment on dasatinib delivery in PDAC¹⁷³. Using this approach (organotypic models consisted of PDAC cells expressing the Src biosensor cultured on top of an ECM gel with embedded primary human fibroblasts), they were able to show that the 3D environment contributes to poor drug delivery to tumour cells, dependent on distance of cells from the invasive edge of the tumour or location relative to vasculature. Furthermore, the group claim that modulating the stroma, using a

combination treatment approach, may facilitate a more effective tumour cell drug delivery.

Despite their elegant approach, there are limitations to this study and results should be further validated. In particular, the subcutaneous *in vivo* models that were used in conjunction with the organotypic models do not simulate adequately the PDAC environment, making comparisons difficult to interpret. In spite of this, the study demonstrates the adaptability of the organotypic model as powerful tool, which can be easily manipulated to address specific questions.

1.5 Evidence of interaction of PSCs and PCCs from *in vivo* studies

Animal models such as xenografts, orthotopic grafts or GEM have enabled the verification that cancer cells interact with PSCs *in vivo*. Early subcutaneous mouse models, in which PSCs and PCCs were injected into the flanks of immunocompromised mice, demonstrated that, in the presence of PSCs, PCC proliferation increased and tumours formed more rapidly than when PCCs were injected alone¹²². However, subcutaneous models do not recapitulate the tumour microenvironment that is so important for PDAC development and are not an ideal model to study metastasis¹⁵⁴. The development of orthotopic and GEM models of PDAC have thus allowed for the study of tumours which form in a more physiologically relevant environment and metastasise to local and distant regions¹¹⁶.

Studies from Apte and colleagues have been pertinent in confirming the role PSCs play a role in regulating PCC behaviour¹⁵⁵. Injection of PCCs (MiaPaCa-2 and AsPC-1 cell lines) in combination with primary human PSC into the mouse pancreas was able to stimulate fibrosis, tumour growth and metastasis. Importantly, no tumours developed in mice injected with PCCs alone. Another group, using an immortalised PSC line, also reported these observed effects of PSCs on PCC growth *in vivo*¹⁷⁴. More recently, elegant sex mismatch studies (injection of male PSCs and female PCCs into the pancreas of female mice) have shown that Y chromosome positive PSCs are able to metastasise through blood vessels, with cancer cells, and seed in distant sites such as liver and the diaphragm, where they are able to facilitate seeding, survival and growth of PCCs¹⁷⁵. In addition to orthotopic mouse models, several GEM models show PDAC tumour development

with a prominent stroma (that contains activated PSCs) and recapitulate human disease. These models include:

Kras^{LSI-G12D/+}; *Trp53*^{LSL-R172H/+}; *Pdx*^{cre/+} (KPC) mice⁸¹

Kras^{LSL-G12D/+}; *Trp53*^{LSL-R172H /+}; *R26*^{LSL-GFP/+}; *Pdx*^{cre/+} (KPGC) mice. These mice have the same activated K-RAS and point mutations in p53 as the KPC mice but also contain enhanced green fluorescent protein (EGFP) cDNA sequence flanked by LoxP sites knocked into the *ROSA26* locus¹⁷⁶.

Kras^{LSL-G12D/+}; *Tgfr2*^{floxlox}; *Ptf1a*^{cre/+} mice. These mice are knockout for type II receptor for TGFβ (*Tgfr2*) specifically in the pancreas and express activated K-RAS in a pancreas epithelium-specific manner. Compared to the p53 inactivation model, PDAC in this model is largely composed of differentiated PDAC, failing to recapitulate the undifferentiated histology that is frequent in human PDAC¹⁷⁷.

A number of preclinical mouse model studies using therapies that target PSCs have shown promise. Enzymatically (PEGPH20) targeting hyaluronic acid (HA, a prominent ECM component abundant in PDAC) in KPC mice was able to restore the tumour vasculature, which permitted high concentrations of Gemcitabine to reach the tumour. In normal tissue, HA provides excellent elasticity to connective tissue, but excessive HA accumulation in solid tumours raises interstitial fluid pressure and compresses blood vessels¹⁷⁸. Targeting HA led to remodelling of the tumour microenvironment, with a significant decrease in activated PSCs, reduced metastasis and increased overall survival¹⁷⁶. In addition, our laboratory demonstrated that treating KPC mice with ATRA significantly decreases tumour growth¹⁷⁰. It is clear that more stromal-targeted therapies are needed. It is therefore imperative that we understand more clearly the mediators of the interactions between PSCs and PCC, which will allow us to identify new therapies that will inhibit

the synergistic effects of PCSs and PCCs. These observations from various groups also suggest the importance of model systems which include the key drivers of desmoplastic reaction is critical to study PDAC development as well as therapy. I aim to use these models to study the most widely known tumour-stroma signalling pathway in human PDAC, FGF signalling.

1.6 Aims

In PDAC, a number of FGFs and FGFRs are over-expressed, correlating with poor patient outcome^{77, 179-185}. For example, over-expression of the IIIC isoform of FGFR1 in PDAC can promote tumourigenesis^{182, 186-188}. Initial experiments exploring FGF2 expression in PDAC described FGF2 apparent in the nuclei of many cancer cells but not in normal pancreatic tissue, suggesting intranuclear FGF2 may be important in this cancer^{77, 185}. However, the function of nuclear FGF2 in PDAC, particularly with respect to PSCs, has not been explored. Thus the first aim of my project was to assess the localisation and expression of FGF2 and its receptor FGFR1 in PDAC tissue. In the second part, I aimed to understand the role and function of nuclear FGFR1 and FGF2 *in vitro* using a panel of pancreatic cancer cell lines as well as an immortalised pancreatic stellate cell line. Finally, I used the 3D organotypic model of PDAC to test assess the hypothesis that blocking nuclear FGFR1 and FGF2 in stellate cells could provide a novel therapeutic strategy in PDAC.

Chapter II MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Cell lines, media and culture reagents.

2.1.1.1 Pancreatic cancer cell lines

Cell line	Origin	Differentiation state	Mutations	Ref
Capan-1	Male age 40 (from liver metastasis)	Well	<i>KRAS</i> , <i>TP53</i> , <i>INK4A</i> , <i>SMAD3</i> and <i>BRCA2</i>	¹⁸⁹
CFPAC-1	Male 26 with cystic fibrosis (from liver metastasis)	Well	<i>KRAS</i> , <i>TP53</i> , <i>SMAD4</i> and methylated <i>INK4A</i> .	¹⁹⁰
Capan-2	Male age 56 (from primary tumour)	Well	<i>KRAS</i>	¹⁸⁹
818.1	Female 75 year old (from ascites)	Well	<i>KRAS</i> , <i>TP53</i> , <i>INK4A</i>	¹⁹¹
COLO-357	From lymph node metastasis	Well	<i>KRAS</i> , <i>SMAD4</i>	¹⁹²
PaTu8898T	From liver metastasis	Well		¹⁹³
AsPc1	Female age 62 (from ascites)	Poor	<i>KRAS</i> , <i>TP53</i> and <i>INK4A</i>	^{194, 195}
MIA PaCa-2	Female age 65 (from primary tumour)	Poor	<i>KRAS</i> , <i>TP53</i> , <i>INK4A</i>	¹⁹⁵
PANC-1	56 year old female (from primary tumour)	Poor	<i>KRAS</i> , <i>TP53</i> and <i>INK4A</i>	¹⁹⁶
PaTu8898S	From liver metastasis	Poor	<i>KRAS</i> , <i>TP53</i> with methylation of the 5'CpG island of <i>INK4A</i>	¹⁹³
Hs766T	46 year old male (from lymph node metastasis)	Poor	<i>KRAS</i> , <i>TP53</i> , <i>SMAD4</i>	¹⁹⁷
SUIT-2	Male 73 (from liver metastasis)	Poor	<i>KRAS</i> , <i>P53</i> , <i>INK4A</i>	¹⁹⁸

Table 2. 1. Shows the human pancreatic cancer cell lines that were used

2.1.1.2 Stromal Cells

Non-tumourigenic, primary and hTERT immortalised human pancreatic stellate cells were used. Using the outgrowth method^{199 200}, pancreatic stellate cells were isolated in an ethically approved manner. The resulting primary cell strains designated PSC1, HPSC and FS1 were verified as being of stellate cell origin based on the expression of characteristic stellate cell markers (expression of cytoskeletal proteins GFAP, Desmin, Vimentin and α SMA) as previously published¹²². An immortalised pancreatic stellate cell line, PS1, was generated previously in the lab, using the outgrowth method followed by immortalisation by expression of ectopic human telomerase reverse transcriptase (hTERT)¹⁹⁹.

2.1.1.3 Pancreatic ductal epithelial cells

Non-tumourigenic HPV16 immortalised human pancreatic ductal epithelium (HPDE) and hTERT immortalised human ductal epithelium (Dec-hTERT) cell lines were used as control pancreatic ductal cell lines^{199, 201}.

2.1.1.4 Culture conditions and routine cell culture

Pancreatic cancer cells were cultured as adherent monolayers in sterile tissue culture flasks (Corning, 431080) in a humidified atmosphere at 37°C, 8% CO₂ in either RPMI (PAA Laboratories E15-840, Capan-1, AsPc1, CFPAC-1), Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories E15-843, HPAF, PANC-1, PaTu8988S, PaTu8988T, COLO-357, MIA PaCa-2, 818.1, Hs766T, SUIT-2) or McCoy's 5A medium modified (ATTC, 30-2007, Capan-2) medium supplemented with 10% foetal bovine serum (FBS, PAA Laboratories A15-104). Pancreatic ductal epithelial cells (Dec-hTERT) were grown in DMEM supplemented with 10% FBS.

HPDE were cultured in keratinocyte serum free medium (Gibco, 10725) supplemented with 0.1mg/ml bovine pituitary extract (BPE Gibco 10450-013) and 5ng/ml epidermal growth factor (EGF, Gibco 13028-014). PS1 and primary stellate cells were grown at the same humidified culture conditions as pancreatic cancer cells in DMEM: F12 medium (Sigma, N6658) supplemented with 10% or 20% FBS, respectively. Puromycin (1µg/ml Sigma, P9620) was added to the medium of PS1 stellate cells as a selection agent.

When cells reached 80-90% confluency, medium was removed and cells were washed with Phosphate Buffered Saline (PBS Sigma PAA17) before trypsin-EDTA (PAA Laboratories, L11003) was added for 1-5 minutes at 37°C in order to detach cells from the flask surface. Once cells were detached, trypsin was inactivated with medium containing 10% FBS. Cell suspensions were centrifuged at 200 x g, followed by removal of supernatant and resuspension of cell pellet in standard medium. If counting of cells was required, 20 µl of cell suspension was pipetted into a haemocytometer prior to centrifugation and cells were counted manually under a light microscope. Cells were subcultured at various ratios (1:2 to 1:5) depending on their growth rate.

For storage of cells, cell pellets were resuspended at various concentrations in a mixture of 90% FBS with 10% dimethyl sulphoxide (DMSO), which acts as a cryoprotectant. One ml of cell suspension was pipetted into a cryovial and cells were slowly frozen first at -80°C to prevent ice crystal formation and then transferred to liquid nitrogen for long-term storage. When recovering cells from liquid nitrogen stock, cells were thawed as quickly as possible in a 37°C water bath. Once thawed, cell suspensions were transferred to a 15ml falcon tube containing prewarmed

standard medium. To remove DMSO, the cell suspension was centrifuged at 1200 rpm for three minutes, the supernatant was removed and cells were resuspended in standard medium and plated onto a tissue culture flask.

2.1.2 Collection of supernatant

For conditioned medium to be used in Western blot analysis, cells were cultured in serum free medium for 12 hours (PS1 cells appeared stressed if starved for longer than this period of time), after which time the medium was collected, filtered and concentrated 20X using centrifugal filter units (Millipore UFC 800324 NMWL 3000) according to the manufacturer's instructions. The membranes used in the centrifugation device are characterized by a nominal molecular weight limit (NMWL); that is, their ability to retain molecules above a specified molecular weight. The device used to concentrate the supernatant in these experiments had a NMWL of 3000 Da. This enabled centrifugation solutes with molecular weights below 3 kDa to be excluded from the membrane and collected in the centrifuge tube while the concentrated supernatant was collected from the filter device sample reservoir. Culture medium (serum free) was used as a negative control. Sample buffer (4X, Invitrogen, NP0007) was added to the concentrated conditioned medium and boiled at 100°C for Western blotting (Section 2.4.5). Similarly conditioned medium (serum free) was collected and filtered from pancreatic cancer cells (COLO357) and normal ductal epithelial cells (Dec-hTERT) to be used in PS1 stimulation assays (Section 2.4.2).

2.2 Reagents

2.2.1 Antibodies

All antibodies are summarised in Table 2. 2

Antibody	Species raised in	Supplier (Cat No)	Dilution for IF or IHC	Dilution for WB
HSC70	Mouse	Santa Cruz (SC7298)	N.A.	1:1000
α SMA	Rabbit	Abcam (ab5694)	1:100	NA
α SMA	Mouse	Dako clone 1A4 (M0851)	1:300	1:100
Vimentin	Mouse	Dako clone V9 (M0725)	c.s 1:2000 FFPE 1:50	1:250
Ki67	Rabbit	Abcam (ab15580)	c.s 1:100 FFPE 1:100	N.A
FGFR1 (recognises intracellular epitope in C terminus)	Rabbit	Santa Cruz (SC121)	N.A	1:1000
FGFR1 (recognises extracellular epitope near transmembrane domain)	Rabbit	Abcam (10646)	c.s 1:100 FFPE 1:500	N.A
FGFR1	Rabbit	Cell Signalling (9740)	N.A	1:1000
Tubulin	Mouse	Sigma (T5168)	N.A	1:2000
Lamin A/C	Goat	Santa Cruz (SC6215)	N.A	1.1000
Actin	Goat	Santa Cruz (SC1615)		
FGF2	Rabbit	Peprtech (500-P18)	N.A	1:500
FGF2	Mouse	Millipore (05-118)	c.s 1:100 FFPE 1:100	N.A
pFRS2	Rabbit	Cell Signalling (38645)	N.A	1:1000
pERK	Rabbit	Cell Signalling (91015)	N.A	1:1000
SC35	Mouse	Sigma (S4045)	1:1000	NA

Antibody	Species raised in	Supplier (Cat No)	Dilution for IF or IHC	Dilution for WB
HSC70	Mouse	Santa Cruz (SC7298)	N.A.	1:1000
α SMA	Rabbit	Abcam (ab5694)	1:100	NA
Cyclin D1	Mouse	Cell Signalling (2926P)	N.A	1:1000
Importin Beta	Mouse	Abcam	1:500	1:1000
Cytokeratin WSS	Rabbit	Dako (Z0622)	1:500	N.A
Anti-mouse HRP	Goat	Dako (P0447)	N.A	1:1000
Anti-mouse HRP	Rabbit	Dako (P0260)	N.A	1:1000
Anti-goat HRP	Rabbit	Dako (P0160)	N.A	1:1000
Fluorescein-goat-anti rabbit	Rabbit	Invitrogen (F27 65)	1:100	N.A
Cy3 donkey anti-mouse	Mouse	Jackson Immunoresearch (715-166-151)	1:100	N.A

Table 2. 2. Antibodies used for experiments

HSC70, heat shock cognate 70 kDa protein 8; α SMA, α -smooth muscle actin; Cytokeratin WSS, wide spectrum screening, recognising a broad spectrum of human cytokeratin; FGFR1, Fibroblast growth factor receptor 1; FGF2, Fibroblast growth factor 2; Lamin A/C HRP, Horseradish peroxidase; c.s, coverslip; FFPE, formalin fixed paraffin embedded; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blot; N.A: not applicable.

2.2.2 PD 173074 FGFR inhibitor

PD 173074 or (N-[2-[[4-(Diethylamino)butyl]amino]-6-(3,5 dimethoxyphenyl)pyrido-[2,3-d]pyrimidin-7-yl]-N'-(1,1-dimethylethyl)urea) (Sigma-Aldrich, P2499), a potent, cell permeable and ATP competitive inhibitor of FGFR²⁰² was dissolved in DMSO to achieve a stock concentration of 20mM. Stock PD 173074 was freshly diluted in culture medium and used at a 2 μ M concentration unless otherwise stated. Medium was changed every two days and cells incubated in culture medium, supplemented with equivalent amount of vehicle (DMSO), served as controls.

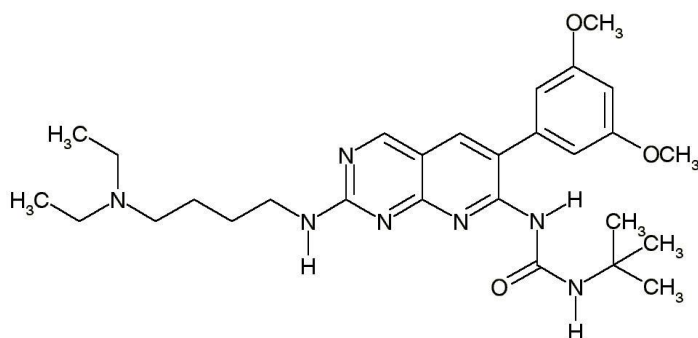


Figure 2. 1 PD 173074

2.2.3 Small interfering RNA (siRNA) oligos

Cells were transfected with a pool of siRNA oligos (Dharmacon CO), whose sequences are listed below in Table 2. 3.

On Target plus SMARTPOOL siRNA FGFR1 L-003131	Target Sequence
J-003131-10	GCCACACUCUGCACC GCUA
J-003131-11	CCACAGAAUUGGAGGCUAG
J-003131-12	CAA AUGCCCUUCCAGUGGG
J-003131-13	GAAAUUGCAUGCAGUGCCG
On Target plus SMARTPOOL siRNA FGF2 L-006695	Target Sequence
J-006695-05	CUAAAUGUGUUACGGAUGA
J-006695-06	UCAAAGGAGUGUGUGCUAA
J-006695-07	GCUAAGAGCUGAUUUUAAU
J-006695-08	GAUGGAAGAUUACUGGCUU
On Target plus SMARTPOOL siRNA Importin beta	Target Sequence
J-017523-06	GAACCAAGCUUGAUCUUGU
J-017523-07	GCUCAAACCACUAGUUUAU
J-017523-08	GACGAGAAGUCAAGAACUA
J-017523-09	GGGCGGAGAU CGAAGACUA
On Target plus SMARTPOOL siRNA FRS2	Target Sequence
J-060152-05	GGUGGGAAGUGCUCGCUUA
J-060152-06	GAGAAGACCUGCACUAUUA
J-060152-07	UGAGAGAACUGCUGCUAUG
J-060152-08	GUACACCGACAGUCUUUAA

Table 2. 3. Sequence of siRNA oligos.

2.3 Cell transfection

2.3.1 *Introduction of siRNA into pancreatic cancer cell lines and pancreatic stellate cells.*

Pancreatic cancer cell lines and pancreatic stellate cells (PS1) were plated into 6 well plates at a confluency of 5×10^4 cells per well (40% confluency) in standard medium containing FBS. The following day the medium was removed from the cell lines and replaced by 1 ml of fresh standard culture medium (without puromycin for the PS1 cells) to achieve the correct final concentration of siRNAs. The cancer and PS1 cells were transfected with a pool of siRNAs at a final concentration of 10 nM, or with a pool of non-targeting siRNA at the same concentration (sequences listed in Table 2.3) using INTERFERin™ (Polyplus 409-10) as a transfection reagent. Transfection complexes were prepared in OptiMEM (Gibco, 3165-027), to which 4 μ l INTERFERin was added, vortexed for 10 seconds and incubated for 10 minutes at room temperature and 105 μ l was added to the cells in culture medium. Subsequently PS1 cells treated with FGFR1 RNAi (24 hours) were harvested, admixed with COLO-357 cancer cells in a 2:1 ratio and cultured in a Miniorganotypic model for seven days (Section 2.4.3.2). Knock down was confirmed by Western blot.

2.4 Functional Assays

2.4.1 *Cell growth assay*

Cancer cell lines and stellate cells were plated into 6 well plates in triplicate at a density of 5×10^4 cells per well and the next day treated with PD 173074 inhibitor (2 μ m) or DMSO as a control. At 24, 48, 72 and 120 hours following treatment, cells were detached with trypsin-EDTA, 500 μ l of cell suspension was added to 9.5 ml of

Isoton and cells were counted with a Casy counter (Sharfe) via electrical signals that are generated when cells pass through a measuring capillary. Cell counts were normalised to the untreated cell number, 24 hours after plating. Each experiment was carried out in triplicate and repeated on three separate occasions. Ki67 staining was also used to identify proliferating cells. Each data point plotted represents the percentage of cells positive for Ki67 per field. Multiple fields were taken per experiment. The total number of cells analysed to obtain this percentage was recorded in the figure.

2.4.2 FGF2 stimulation assays

For inhibitor experiments, PS1 cells were plated in 6 well plates at a density of 3×10^5 cells per well, serum starved for 12 hours and then treated in serum free media for one hour with FGFR inhibitor PD 173074 (2 μ M) before stimulation with 100 ng/ml recombinant FGF2 (PeproTech, 100-18C) and 300 ng/ml heparin sodium salt for different time points. PS1 cells were then lysed and used for Western blot analysis (Section 2.4.5). For quantification of nuclear FGFR1 and FGF2 following FGF2 stimulation, PS1 cells were plated on coverslips in 12 well plates at a density of 1×10^5 cells per well, serum starved for 12 hours before treating with 100 ng/ml recombinant FGF2 and 300 ng/ml heparin sodium salt for various time points. PS1 cells were then fixed and stained according to Section 2.5.2. Similarly, conditioned medium (serum-free) from normal or cancer cells was added to serum starved PS1 cells for 6 hours before cells were fixed and stained for quantification of nuclear FGFR1 and FGF2.

2.4.3 Organotypic culture

2.4.3.1 Air-liquid interface organotypic model

Figure 2. 2 shows the organotypic model used as described before¹⁶⁵. In order to study the invasion of cancer cells in a 3D model, an air liquid interface model was used. An extracellular matrix (ECM) equivalent, composed of 75% Collagen type I (BD Biosciences 354236) and 25% Matrigel (BD Biosciences 354234) was prepared on ice by mixing one ml of a mixture of 5.25 volumes Collagen type I, 1.75 volumes Matrigel, 1 volume of 10X DMEM (PAA Laboratories E15-843), 1 volume of DMEM and 1 volume of filtered FBS, was plated into 24 well plates coated with diluted Collagen type I (1:100 in PBS). Gels were made in triplicate. Once the gels had polymerised, 1.7×10^5 cancer cells mixed with 3.3×10^5 stellate cells, or 5×10^5 cancer cells alone (control) were added onto the gels in 1ml of medium and left to adhere overnight at 37°C. The next day, the gels were lifted onto a metal grid covered by a nylon membrane precoated with 7 volumes Collagen type I, 1 volume 10 X DMEM, 1 volume DMEM and 1 volume FBS. 250µL of the mixture was pipetted onto the nylon membrane and allowed to polymerise for 15 minutes at 37°C, cross-linked with 1% glutaraldehyde /PBS and left for one hour at 4°C. Glutaraldehyde was removed by washing the membrane three times with PBS and once with medium, then covering in medium and leaving overnight at 4°C. The following day, the submerged organotypics were raised to grids and fed from below with medium supplemented with PD 173074 (2 µM) or DMSO (control). Medium was changed every other day and gels were harvested at 14 days following treatment, fixed in 10% neutral buffered formalin, bisected and embedded in paraffin.

2.4.3.2 *Mini organotypics*

Mini organotypics were constructed using primary PSCs (PSC1) in 0.4 μ M Transwell inserts (Corning, 3413) in order to accommodate for the limited availability of primary PSC cells. Figure 2. 2 shows the construction of the mini organotypic model. First, the bottom of the insert was coated with Collagen type I (1:100 dilution in PBS) and left at 37°C for one hour. Following this, 120 μ l of ECM equivalent, consisting of 5.25 collagen type I, 1.75 Matrigel, 1 volume of 10X DMEM (PAA Laboratories E15-843), 1 volume of DMEM and 1 volume of filtered FBS, was plated into inserts and left to polymerase at 37°C for 2 hours. When the gels had polymerised, 200 μ l of PSCs (0.66×10^5 cells) and COLO-357 cells (0.33×10^5 cells) admixed or COLO-357 cells alone (1×10^5 cells) were plated on to the gels and 600 μ l of medium (DMEM) was added to the bottom of the well. At this point gels were left overnight at 37°C for cells to adhere. The next day medium in the bottom of the well was replaced with DMEM (10% FBS) containing either PD 173074 (2 μ M) or DMSO (control). Similarly, mini organotypics were constructed as above using PS1 cells treated with scrambled or FGFR1 RNAi (24 hours) and COLO-357 cells in a 2:1 ratio. For these organotypics, DMEM (10% FBS) medium was used in the bottom of the well. Medium was changed on alternate days and the organotypics were harvested at day seven, fixed in 10% neutral buffered formalin, bisected and embedded in paraffin. Twelve high power fields (HPF) were counted for each organotypic gel and an average of these fields were plotted and are represented by one data point. Nine gels were analysed from three separate experiments. Alternatively, to quantify invasion in the Mini organotypic model in which PS1 cells were treated with FGFR1 RNAi, total cell number invading per gel was plotted.

2.4.4 Chromatin Immunoprecipitation (ChIP)

PS1 cells were plated onto 15 cm dishes (total ~ 10×10^6). The next day, chromatin was cross-linked by adding using 1/10 volume of freshly-prepared formaldehyde Solution (11% formaldehyde Sigma F-8775, 0.1M NaCl, 1mM EDTA, pH 8, 50mM HEPES, pH 7.9) to the existing media for 15 minutes at room temperature. To stop the fixation 1/20 volume glycine (2.5M, Sigma G-7403) solution was added to the existing media in each container and left at room temperature for 5 minutes. Cells were scraped, using a rubber policeman, thoroughly from the culture surface and centrifuged to pellet the cells. Cells were washed twice using chilled PBS-NP-40 (0.5%) and centrifuged once more to pellet the cells. Finally, the cells were centrifuged a third time, the supernatant was removed completely from the cell pellet, and cell pellets were snap frozen on dry ice and stored at -80°C , before being shipped to Active Motif (Carlsbad, CA). ChIP reactions were carried out using 30 μg of PS1 cell chromatin and anti-FGFR1 antibody from either Abcam (ab10646) or Cell Signaling (9740). The ChIP DNAs were processed into a standard Illumina ChIP-Seq library and were sequenced on MiSeq to generate >2 million reads. About 5 million reads per sample were aligned to the human genome (hg19), and after removal of duplicate reads, 2.4 and 1.4 million alignments were obtained for the Abcam and Cell Signaling sample..

2.4.5 Western blotting

2.4.5.1 Isolation of protein

Two methods were used to isolate protein from cell lines. To determine protein concentration of cell lysates, cells were grown to 80% confluency, washed with ice

cold PBS and lysed on ice with RIPA lysis buffer (Upstate 20-188) with freshly added protease inhibitor (Calbiochem 539131) and phosphatase inhibitor cocktails (Calbiochem 524625) at a dilution of 1:100. Cells were collected in a 1.5 ml Eppendorf, by scraping with a rubber policeman, and left for 20 minutes on ice with agitation (vortexing) every 10 minutes. Cell debris was collected by centrifugation of the lysates at 20,000 x g for 15 minutes at 4°C. Protein concentration was determined with a Bio-Rad DC protein Assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Following determination of protein concentration, sample buffer (4X, Invitrogen, NP0007) was added to the lysate in order to prevent protein degradation and, if lysates were not to be used immediately, samples were stored at -80°C. If the concentration of protein was not required (for example, the same cell line was plated at equal densities and subjected to the same treatment, or the treatment did not affect cell growth), cells were lysed at room temperature using the same amount of sample buffer and homogenised briefly by sonication.

2.4.5.2 Western blot analysis

Cell lysates were boiled at 100°C for 5 minutes to denature proteins before brief centrifugation to collect contents. Equal amounts of denatured protein (15-25 µg) were loaded onto 4-12% NuPage Bis-Tris pre-cast gels (Invitrogen NP0335 or NP0336) and transferred to nitrocellulose membrane (GE Healthcare, PA1000). Transfer efficiency and protein loading was confirmed using Ponceau S (Sigma, P7170), a negative stain, which binds to the positively charged amino groups of protein on the nitrocellulose membrane. Membranes were then washed with PBS and non-specific binding was blocked by incubation with 5% milk, followed by incubation with primary antibody in 3% BSA/TBS at 4°C overnight. Membranes were washed with 0.1% Tween20-TBS and subsequently incubated with secondary HRP-

conjugated antibody for one hour at room temperature. Specific protein bands were visualised using an Amersham ECL Western Blotting Detection Kit (GE Healthcare, RPN2106) and photographic film (Fujifilm, 100NIF).

Concentrated supernatant samples, collected from cells seeded at equal densities, were diluted in sample buffer, boiled at 100°C and treated as described above. Equal loading was determined by staining the membranes with Ponceau S before blocking with milk and addition of primary antibody.

2.4.5.3 Stripping membranes

Membranes were stripped using Reblot plus mild (Millipore 2502) for 10 minutes, washed with PBS and blocked with 5% milk/TBS before reprobing with primary antibody as above.

2.4.5.4 Densitometry and analysis

For Western blotting, densitometric analysis of specific bands was carried out using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2005.) to obtain a semi-quantitative measurement of the level of total protein, band densities were normalised to the loading control housekeeping proteins (Actin, HSC70 or Tubulin) on the same membrane.

2.4.6 Nuclear fractionation.

For cell fractionation, PS1 or COLO-357 cells were plated on 10 cm plates (Corning, 430167) and experiments were performed according to manufacturer's instructions

(Nuclear Extraction kit, Imgenex, 10081K). Sample buffer (4X, Invitrogen, NP0007) was added to the lysates and treated as above (Section 2.4.5.2). The fraction purity was confirmed (Lamin A/C and Tubulin antibodies for the nuclear and cytoplasmic fractions, respectively)

2.5 Immunofluorescence

2.5.1 Patient samples

Ethically approved human PDAC samples were arranged in tissue micro-arrays as described before ^{169, 203}.

2.5.2 Cells cultured on coverslips

Cells were seeded onto 13 mm diameter coverslips in a 12 well or 6 well plate (plating onto a 6 well plate allowed for isolation of protein at the same time so as to treat all cells equally) as a monoculture at a density dependent on planned time of fixation. For fixation the following day, 3×10^5 cells were plated onto 6 well plates, whereas for cells plated onto coverslips in a 12 well plate they were seeded at 1×10^5 per well.

For immunofluorescence, cells were fixed with 4% formaldehyde, permeabilised with 0.1% saponin/PBS, blocked with 6% BSA and incubated for one hour at room temperature with primary antibody followed by appropriate fluorescently labelled secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, P36931). Negative controls were incubated with isotype specific immunoglobulins at the same concentration as the primary antibody used.

2.5.3 Paraffin Embedded gels and patient tissue

For immunofluorescent staining of paraffin embedded gels and patient tissue, 4 μ M sections were dewaxed and rehydrated. Unless stated, antigens were retrieved by boiling sections in 10mM citrate buffer (pH 6.0) for 20 minutes. Sections were blocked with 6% BSA/PBS and incubated with primary antibody overnight at 4⁰C, washed in PBS and incubated for one hour at room temperature with the appropriate secondary antibody. Sections were washed in PBS and then Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, P36931). Negative controls were incubated with isotype specific immunoglobulins at the same concentration as the primary antibody used.

2.5.4 Co-localisation

Double-stained images were taken using a confocal laser scanning microscope (Zeiss LSM 710, Carl Zeiss, Germany) and thresholds for each channel of interest were set to correct for background fluorescence. Co-localisation of two proteins appeared as white pixels. Nuclear FGFR1 and FGF2 co-localisation in PDAC tissue microarrays (TMAs) was quantified by counting the total number of stromal fibroblasts and cancer cells per field, and then assessing the percentage of those cells that showed co-localisation of FGFR1, FGF2 and DAPI (white pixels) with constant pre-set thresholds. For patients in whom both FGFR1 and FGF2 were scored (36 patients) correlation between the presence of FGF2 and FGFR1 in the nuclei of stromal fibroblasts was assessed. The same technique was used for *in vitro* cellular co-localisation of FGFR1 and FGF2.

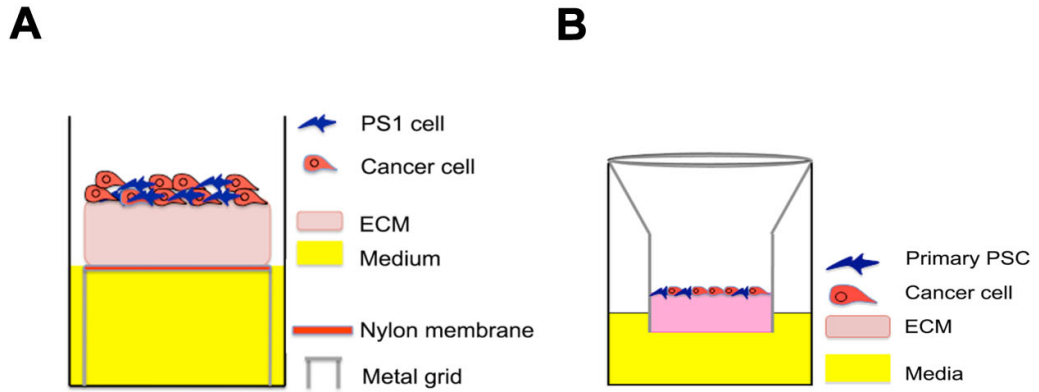


Figure 2. 2 Schematic model of a raised air liquid and Mini-organotypic culture model.

A. Submerged ECM gels were raised onto a metal grid coated with a nylon membrane and fed from below with medium containing either PD 173074 (2 μ M) or DMSO vehicle control. PS1 cells were admixed with cancer cells in a 2:1 ratio and cultured on top of the ECM. Cultures were harvested at day 14. B. Schematic model of Mini-organotypic culture model. Transwell inserts (0.4 μ m) were coated with Matrigel and Collagen. Cancer cells (COLO-357) were admixed with primary PSCs or PS1 cells (treated with FGFR1 or scrambled RNAi) in a 1:2 ratio and cultured on top of the ECM. Cultures were fed from below with DMEM (10% FBS) or DMEM (10% FBS) supplemented with either PD 173074 (2 μ M) or DMSO vehicle control. Cultures were harvested at day 7.

2.6 Statistical analysis

All quantitative data are presented with respective statistical tests dependent on normality of distribution and significance was defined as $p < 0.05$, as analysed in prism v 5.03 (Graphpad, USA). Unless otherwise stated, all experiments were performed independently a minimum of three times.

2.6.1 *Functional assays and western blot*

For normally distributed data, groups were compared using two tailed Student's t-test. Significance was defined as $p < 0.05$.

2.6.2 *Immunofluorescence*

For all experiments where pixel intensity was analysed, 3 separate experiments were carried out and at least four random fields were analysed (~10 cells per field for PS1 and ~ 30 for cancer cells). Total FGF2, nuclear FGF2 and FGFR1 levels were quantified with Image J software. Images were taken at x630 magnification and the area of red or green stain within the region of interest was determined. Initial thresholds were set and kept constant for all images analysed. Data were compared and analysed using two tailed Students t-test or ANOVA (Kruskal Wallis with Dunn's multiple comparison test) and significance was defined by $p < 0.05$. Multiple fields (at least 3) were taken per experiment and the total cells per field were analysed. An average of total, nuclear FGFR or FGF2 per field was plotted. All experiments were performed on three separate occasions. The total number of cells analysed is recorded in each figure.

To analyse the percentage of cells with nuclear FGFR1 and FGF2 in organotypic sections, multiple fields per gel were analysed. The average percentage of PS1 cells across these fields with nuclear FGFR1 or FGF2 were plotted and are shown by each graphical data point. Nine gels were analysed in total from three separate experiments. In order to analyse the percentage of nuclear FGFR1 and FGF2 positive fibroblasts at the invasive front or centre of the tumour in tissue sections, several fields per section per patient (four patients) were analysed. Each data point is representative of one HPF. The total number of fibroblasts analysed for all the patients is recorded in the figure.

CHAPTER III RESULTS - PART I

Analysing the expression and localisation of FGFR1 and FGF2 in human pancreatic ductal adenocarcinoma.

3.1 Introduction

Many critical embryonic signal transduction pathways, quiescent in the adult pancreas, are re-activated during PDAC progression. One such pathway is the FGF family, members of which are deregulated in pancreatic cancer. FGFs and their receptors drive a number of important developmental signalling pathways, so it is not surprising that cancer cells may hijack this pathway. Indeed, the link between aberrant FGFR signalling and tumorigenesis is striking. There is now evidence from multiple cancer types that suggests FGF signalling acts in an oncogenic context, driving proliferation, survival, migration, invasion and angiogenesis²⁰⁴. Indeed, the importance of FGF signalling in tumour pathogenesis was highlighted by a screen of more than 1000 somatic mutations found in the coding exons of 518 protein kinase genes from over 200 different cancers. Of the non-synonymous mutations, FGF signalling was one of the most commonly mutated pathways²⁰⁵.

Activating mutations in FGFR3 have been identified in over 50% of bladder cancers, as well as cervical cancers, multiple myeloma, prostate cancer and spermatocytic seminomas²⁰⁶. Mutations in FGFR2 have also been described in a small proportion of endometrial carcinomas, which are highly sensitive to FGFR kinase inhibitors, suggesting FGFR2 oncogenic addiction²⁰⁷. *FGFR2* is amplified in around 10% of gastric cancers while *FGFR1* amplification is commonly associated with oestrogen

positive breast cancer²⁰⁸ and to a lesser extent in oral squamous carcinoma, ovarian carcinoma, bladder cancer and rhabdomyosarcoma²⁰⁹⁻²¹¹. Indeed, amplifications of *FGFR1*, reported in >10 % of breast cancer patients, are associated with a poorer outcome^{212, 213}. In particular, amplification and subsequent overexpression of *FGFR1* contributes to poor prognosis in luminal-type breast cancers, providing a mechanism for resistance to endocrine therapy²¹⁴. Furthermore *FGFR1* amplification is the strongest independent predictor of poor outcome in patients with ER-positive tumours²¹³.

FGFs are up regulated in approximately 60% of all PDAC samples. Previous studies have demonstrated that human PDACs express increased levels of FGF2 and its receptor *FGFR1* as compared to normal human pancreatic tissues, using immunohistochemical staining, northern blotting and *in situ* hybridisation. Other studies have correlated the degree of FGF2 expression with advanced tumor stage and shorter patient survival at the time of diagnosis⁷⁷. PDAC cells express high levels of the mesenchymal *FGFR1* c isoform, which signals through the MAP- and Jun-kinase pathways to affect changes in cell proliferation, adhesion, and/or motility¹⁸². However, further studies are required to fully understand how FGF ligands, which activate different receptor isoforms on cancer cells, are orchestrated to induce a malignant phenotype in pancreatic cancer. Despite this, results suggest FGF signalling is an important mediator of PDAC and may contribute to the intense desmoplasia, as elevated levels of FGF2 are associated with this phenotype in primary tumours²¹⁵. However, despite many studies, the mechanism by which *FGFR* signalling might control metastatic cell behavior and contribute to cancer progression in PDAC is far from clear. Initial experiments exploring FGF2 expression in PDAC described FGF2 as being apparent in the nuclei of many

cancer cells but not in normal pancreatic tissue, suggesting intranuclear FGF2 may be important in this cancer and that FGF2 may exert differential effects in the milieu of the tumour microenvironment^{185, 216}. However, the function of nuclear FGF2 in PDAC has not been explored. Thus my first aim was to determine the expression and localisation of FGF2 and FGFR1 in human PDAC.

3.1.1 Nuclear FGFR1 and FGF2 in human PDAC

The availability of in house PDAC tissue microarrays enabled me to analyse expression of FGFR1 and FGF2 in a number of PDAC patient samples. Cell-specific expression of FGF2 and FGFR1 in human PDAC was assessed by double staining (FGF2/cytokeratin, FGFR1/vimentin or FGFR1/ α SMA) PDAC tissue microarrays. Analysing tissue from 46 patients demonstrated that FGF2 was expressed universally in PDAC tissue. In contrast to the cytoplasmic expression of FGF2 in cancer cells, many (~35%) myo-fibroblasts (activated PSCs¹⁰⁵) expressed nuclear FGF2 (Figure 3.1). The confocal cross-hair function is a powerful tool for visualising comparison and quantification of colocalisation of across multiple fields (Figure 3.2). This method specifically defined colocalisation of FGF2 and DAPI, as depicted by white pixels. The same method was also used to quantify colocalisation of FGFR1 and DAPI. Immunostaining with antibodies to FGFR1 and vimentin (to depict the stroma) revealed nuclear and cytoplasmic staining of FGFR1 in ~39% of cancer cells and ~37% of myo-fibroblasts (Figure 3.1). The specificity of FGFR1 and FGF2 staining was shown by the lack of immunoreactivity using matched isotype immunoglobulins (Figure 3.1). Furthermore, taking those patients who were successfully scored for nuclear FGFR1 and FGF2 (36 patients), I found a positive correlation of nuclear FGF2 and FGFR1 in myo-fibroblasts, but not in cancer cells

(Figure 3.2). Thus FGFR1 and FGF2 show significant colocalisation in activated myofibroblasts but not in tumour cells.

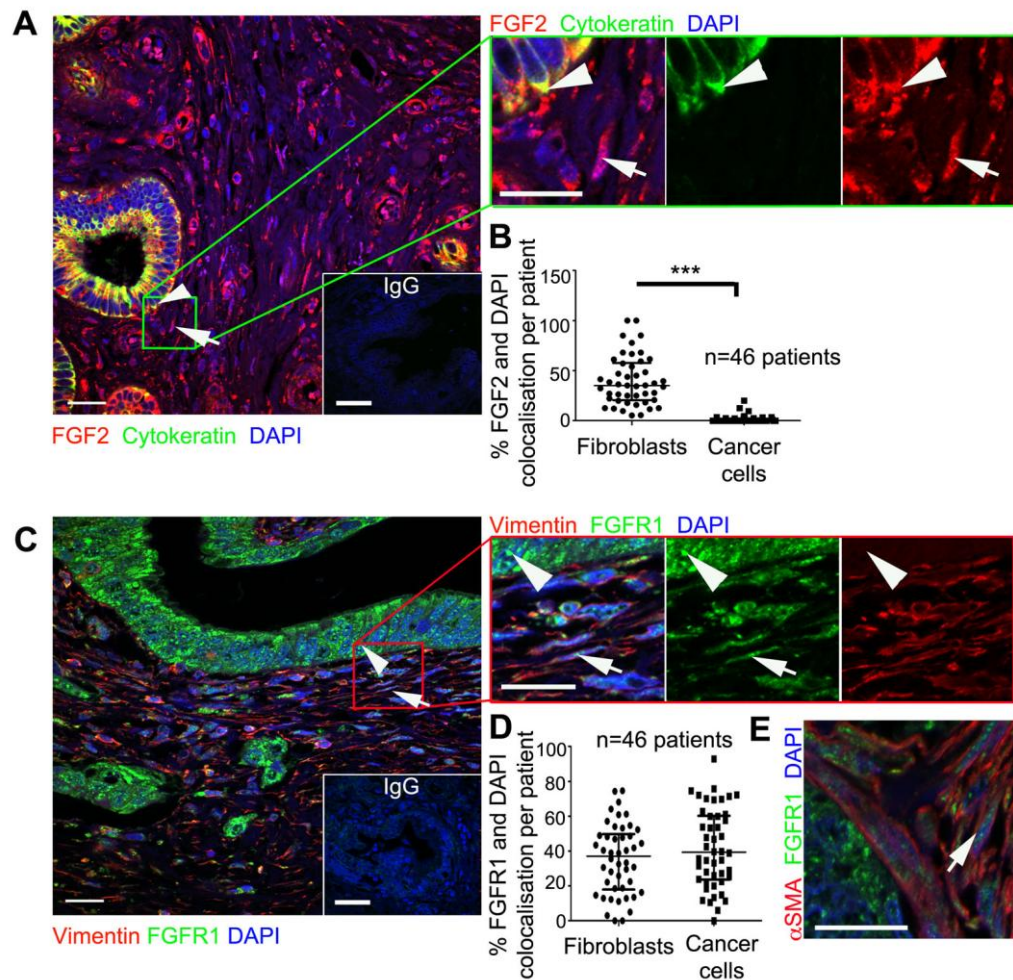


Figure 3. 1 FGF2 and FGFR1 localise in the nucleus of activated fibroblasts in human PDAC tissues.

(A) PDAC tissue showed cytokeratin positive (green, arrowhead) epithelial tumour cells with cytoplasmic FGF2 (red); however, cytokeratin negative stromal cells with fibroblastic morphology (arrow) showed nuclear FGF2 staining (red), demonstrated clearly in the side panel. DAPI stains the nuclei. Inset box shows IgG control. (B) FGF2 and DAPI pixel co-localisation analysis (of 46 patients, 1 TMA core analysed per patient) performed by confocal microscopy confirmed the presence of nuclear FGF2 in 35% of stromal cells but not in tumour cells. (C) Similarly, FGFR1 (green) was present in the nuclei of fibroblasts, as identified by vimentin expression (red, arrow). Vimentin negative cells with epithelial, glandular morphology showed cytoplasmic and nuclear FGFR1 (arrow head) as shown in side panel consistent with A. Inset box shows IgG control. (D) FGFR1 and DAPI pixel co-localisation analysis performed (46 patients, 1 TMA core analysed per patient) by confocal microscopy, as above, confirmed presence of nuclear FGFR1 in 37% of stromal cells as well as 39% of cancer cells. (E) Results in C and D were confirmed by independent co-staining of serial sections with α SMA (red) and FGFR1 (green). Scale Bar: 20 μ m, IgG 100 μ m. *** p <0.001, Mann-Whitney U-test (B,D). Data summary represented by median \pm interquartile range.

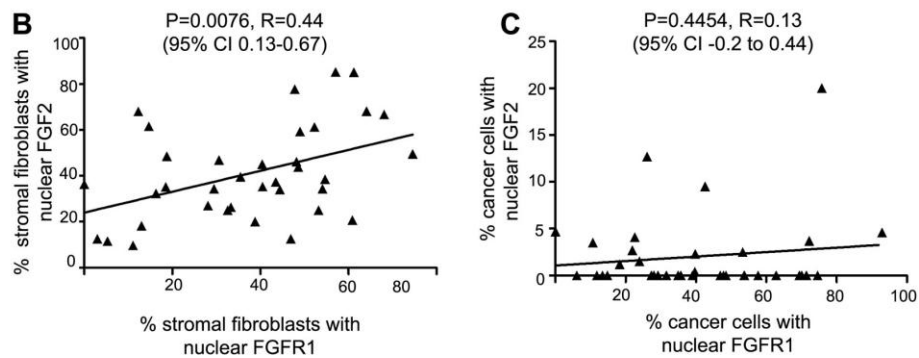
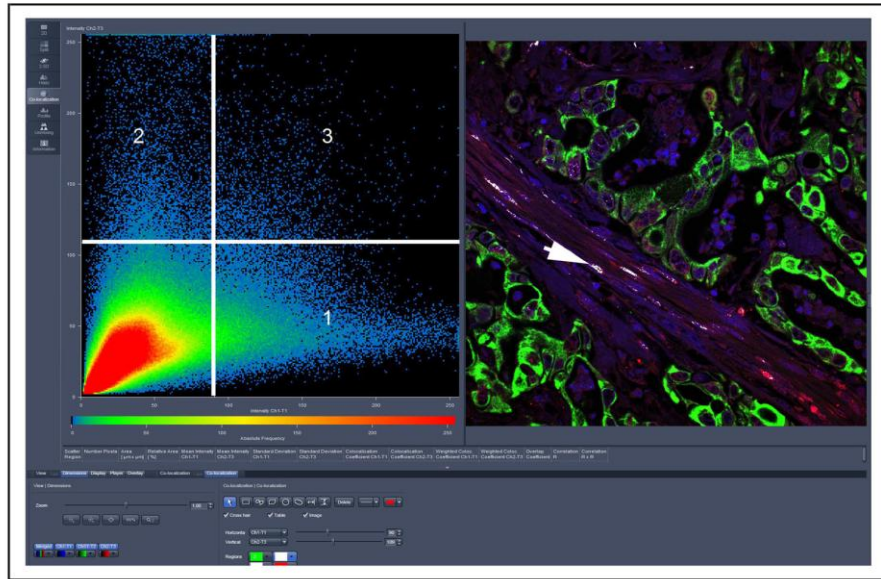
A

Figure 3. 2 Nuclear signal co-localisation with confocal microscopy

(A) Human pancreatic tissues were stained with antibodies to FGF2 (red) and cytokeratin (green) and counter-stained with DAPI (blue). Using the confocal cross-hair function is a powerful tool for visualising colocalisation of a chosen region or field, the automated method computes each channel pixel intensities as well as background intensities. This then leads to the defined distribution of all image pixels over the four cross-hair quadrants of the scatter plot with the background pixels sorted to the bottom left quadrant. The single channels are then displayed in quadrant 1 and 2 and pixels having intensity above the background in both channels, i.e co-localised pixels are represented in quadrant 3. The image pixels corresponding to quadrants 1, 2 and 3 of the scatter plot are colour coded to allow identification of co-localised regions. Thresholds for the crosshair are set and kept constant to allow comparison and quantification of colocalisation of across multiple fields. This figure demonstrates the specificity of this method in defining colocalisation of FGF2 and DAPI as depicted by white pixels. The same method was also used to quantify colocalisation of FGFR1 and DAPI (B) Significant correlation was found between the presence of FGF2 and FGFR1 in the nuclei of stromal fibroblasts from 36 patients who had been scored for both FGFR1 and FGF2, however there was no correlation between percentages of cancer cells with nuclear FGF2 and FGFR1 (C). Each data-point represents one patient.

3.1.2 Nuclear FGF2 and FGFR1 at the invasive front of human PDAC

The most predominant activated fibroblasts in PDAC are PSCs, which can promote tumour growth and invasion^{121, 217}. Thus it was possible that the fibroblasts in which FGFR1 and FGF2 co-localised in the nucleus were activated pancreatic stellate cells, and nuclear localisation of FGFR1 and FGF2 may regulate a more invasive phenotype. I therefore chose to examine whole sections from human PDAC and compared nuclear FGFR1 and FGF2 in fibroblasts at the invasive front of the tumour (invasion into tissue such as duodenum, adipose or normal pancreas) to those at the centre of the tumour. Independent immunostaining and analysis of FGFR1 and FGF2 revealed few fibroblasts with nuclear FGFR1 or FGF2 positivity (~ 20%) at the centre of the tumour. However, upon examination of areas of tumour invasion, there were significantly more fibroblasts with both nuclear FGFR1 and FGF2 (~ 45 to 50%, respectively) (Figure 3. 3 and Figure 3. 4). This suggests a possible role for nuclear FGFR1 and FGF2 in driving PDAC tumour invasion *in vivo*.

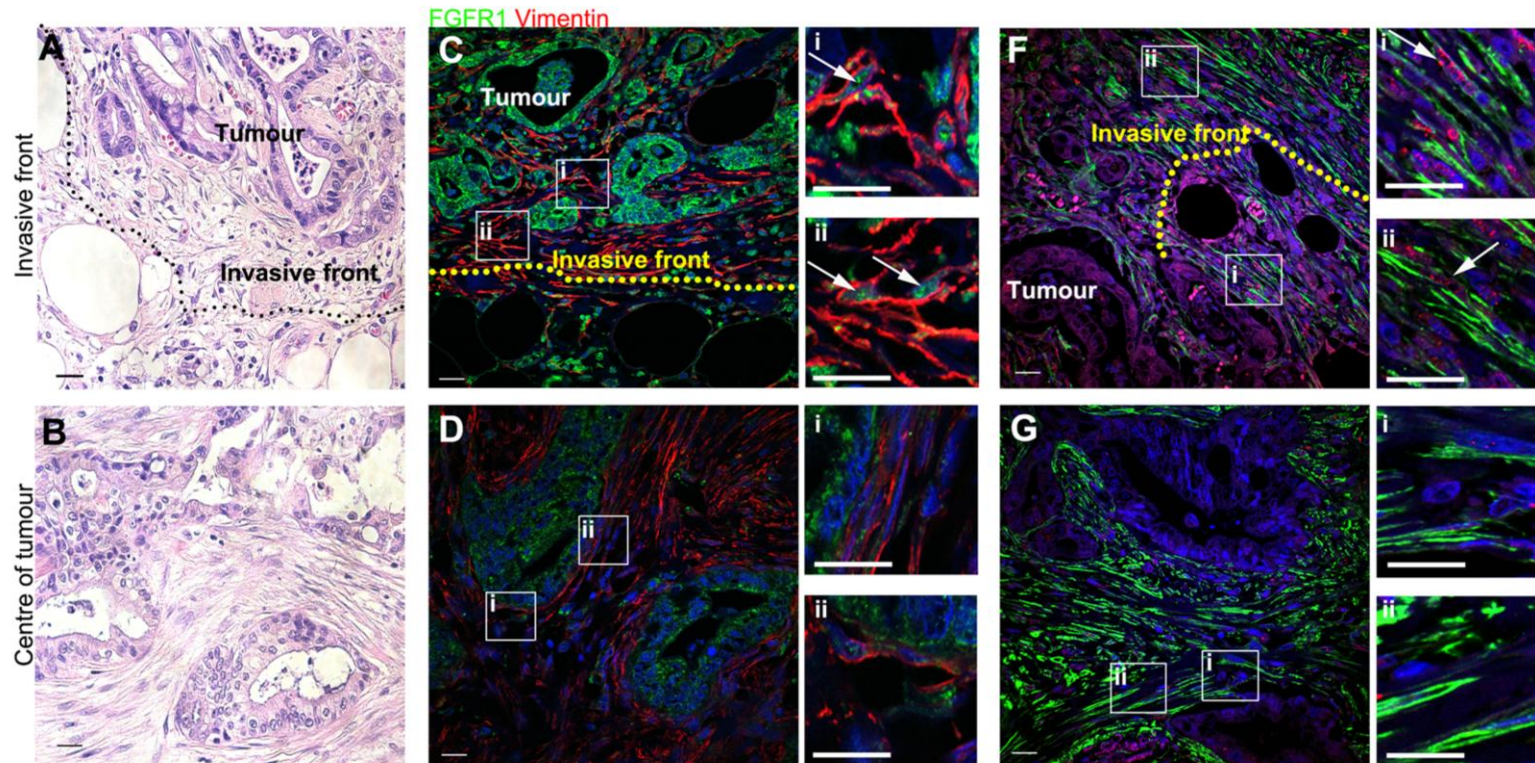


Figure 3.3 Fibroblasts with nuclear FGFR1 and FGF2 are abundant at the invasive front of the tumour

Fibroblasts (vimentin positive, red) invading adipose tissue (invasive front demarcated in A, C and F) in PDAC sections showed increased nuclear FGFR1 (green) relative to those at the centre of the tumour (D) (magnification of boxed areas which represent stromal fibroblasts are shown in Ci, Cii, Di and Dii). Staining of serial sections of the same tumour as in C revealed that a significantly higher number of myo-fibroblasts (α SMA positive, green) invading adipose tissue in PDAC sections (F) showed nuclear FGF2 (red), compared to those at the centre of the tumour (G) (magnification of boxed areas, which represent stromal fibroblasts, are shown in Fi, Fii, Gi and Gii). Scale Bar: 20 μ m.

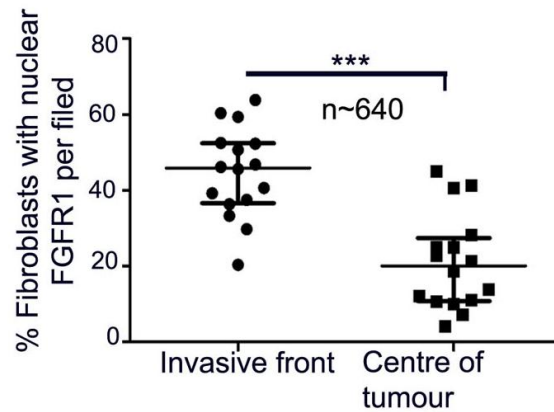
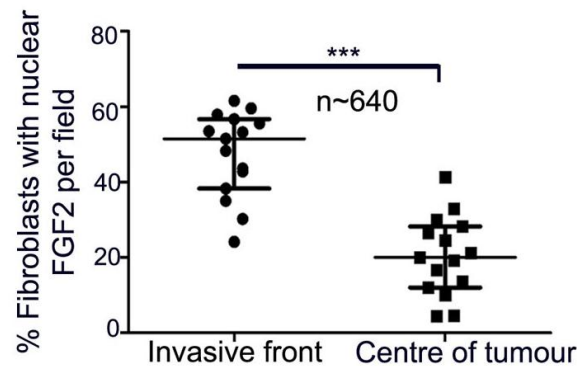
A**B**

Figure 3. 4 increased proportion of fibroblasts at the invasive front display nuclear FGFR1 and FGF2, compared to the centre of the tumour

Quantification of PDAC patient sections showed that a significantly higher number of fibroblasts at the invasive edge of the tumour (invading adipose, normal tissue or duodenum) had nuclear FGFR1 (A) and FGF2 (B), compared to those fibroblasts close to the centre of the tumour. Each data point represents the percentage of fibroblasts (vimentin or α SMA positive) with nuclear FGFR1 or FGF2 per field. Several fields at the invasive front or centre of the tumour were quantified per patient. Stromal fibroblasts were analysed in four patients (n=4, ~640 fibroblasts at the invasive front and central tumour were counted in total).

*** $P < 0.001$. Mann Whitney U-test. Data summary represented by median \pm interquartile range.

3.2 Discussion.

Immunostaining and colocalisation analysis show that both FGF2 and FGFR1 colocalise to the nucleus exclusively in PSCs *in vivo* (human PDAC), which suggests that this receptor and ligand may play an important role in regulating stromal fibroblast behaviour. These findings correlate with earlier studies that report intense FGF2 staining in the stromal fibroblasts in PDAC tissue²¹⁸. Interestingly, in these same studies that showed patients with strong stromal FGF2, adjacent tumour cells were negative for FGF2. However, the importance of nuclear FGF2 staining in the stroma of these tumours has never been explored. The observation that FGFR1 and FGF2 localise to the nucleus in fibroblasts in PDAC identifies a potential novel mechanism by which FGFR1 signalling regulates stromal cell behaviour. Furthermore, examination of PDAC sections revealed that a significant percentage of myofibroblasts at the invading tumour front displayed nuclear FGFR1 and FGF2, compared to myofibroblasts in the central core of the tumour, emphasising the possible role of nuclear FGFR1 and FGF2 in driving PDAC tumour invasion.

FGF2 has been implicated in tumour angiogenesis through its ability to stimulate the growth of endothelial cells. In addition, FGF2 can stimulate fibroblast and epithelial cell growth^{219, 220}. Classically, FGF2 mediates its effect by binding to a cell surface FGFR containing an intracellular tyrosine kinase domain²²¹ (to be discussed in further detail in section IV), however the presence of the ligand in the nucleus of fibroblasts in the stroma of PDAC suggests specific nuclear functions for this molecule in addition to its capacity to signal at the cell surface. Given that PDAC tumours are hypovascular, the major role of FGF2 is unlikely to play a role in stimulating angiogenesis. FGF1, -2, and -3 have all been detected in the cell nucleus, where their presence is likely to be important for their function²²².

Internalisation of FGF1 is essential for stimulation of cell division and a dual mode of action has been proposed²²³. FGF2 nuclear localisation is associated with proliferation; proliferating astrocytes show predominantly nuclear FGF2, whereas in contact-inhibited astrocytes FGF2 is mainly cytoplasmic²²⁴. FGFR-1 and FGFR-4 have also been detected in the nucleus of human glial cells and chondrocytes, and liver cells, where receptor accumulation appears to coincide with cell proliferation^{225, 226}. Furthermore, FGFR3 accumulates in the nucleus of human breast cancer cells but not normal breast epithelia *in vivo*, and this may be stimulated by stromally released FGF1 in breast cancer tissue²²⁷. Thus, nuclear FGFR3 may play an important role in the progression of breast cancer.

While the clinical significance of nuclear FGFR1 and FGF2 has not been explored, nuclear localisation of RTKs has been implicated in poor prognosis in a variety of studies²²⁸⁻²³¹. The EGFR family of RTKs, comprising EGFR (Erb-1/HER-1), ErbB-2 (HER-2/neu), ErbB-3 (HER-3) and ErbB-4 (HER-4), provides the best studied examples of nuclear growth factor receptors that play a role in driving distinct biological roles. Nuclear EGFR was first observed in hepatocytes during liver regeneration²³². In addition, EGFR ligands including EGF and pro-transforming growth factor α were also found in the nucleus of proliferating hepatocytes²³³. In response to stimulation, activated EGFR translocates to the nucleus and the C-terminus of EGFR, containing a proline-rich sequence (typical feature of a transactivation domain for transcription factors), interacts with the AT-rich response sequence within the promoter of *Cyclin D1*²³⁴. EGFR alone can activate the *Cyclin D1* promoter, however it interacts with transcription factors including STAT3 to activate a number of cancer promoting genes such as *iNOS* and *Twist*, STAT5 to activate *Aurora* and E2F1 to regulate *B-Myb*^{229, 235, 236}. EGFR can interact with STAT3 to activate COX 2 expression in glioblastoma cells²³⁷. In breast cancer cells,

nuclear ErbB-3 transactivates the COX2 gene by interacting with a HER-2 associated sequence and increasing the expression of COX2 in breast cancer cells²³⁸.

These *in vitro* observations have been investigated in clinical practice, and may be relevant to the understanding of the role of nuclear FGFR and its ligands in the context of PDAC development. Indeed, an increased expression of nuclear ErbB4 and EGFR correlates with disease progression in breast cancer patients²³⁹. Treating lung cancer cells with low doses of radiation can stimulate EGFR to go to the nucleus and, recently, it has come to light that drug resistance can correlate with an increase in nuclear EGFR in non-small cell lung cancer cells²⁴⁰. This observation was linked to the EGFR-dependant increases in the transcription of *BCRP/ABCG2*, a gene which encodes a multi drug resistance pump²⁴¹. Furthermore, studies of other growth factors have shown that nuclear accumulation of the full length type 1 insulin like growth factor 1 receptor (IGF1R) is a predictor of poor prognosis in clear cell renal cancer²⁴². The presence of lymph node metastasis in patients with hepatocellular carcinoma is associated with a low probability of survival and the occurrence of regional lymph node metastasis is a prognostic factor that governs choice of therapy for patients²⁴³. Recently, Ziang and colleagues have shown that nuclear expression of CXC chemokine receptor 4 (CXCR4), a rhodopsin like G protein coupled receptor, is a risk factor for developing lymph node metastasis and correlates with poor patient outcome in hepatocellular carcinoma²⁴⁴. Thus, it is also possible that, in PDAC, the degree of nuclear accumulation of FGFR1 and FGF2 could be used as a predictor of how a patient may be treated and may warrant further investigation in a larger cohort of patient samples.

A hallmark of PDAC is its dense desmoplastic reaction. This desmoplasia is complex,

consisting of fibroblasts and proliferating stellate cells, that produce and deposit fibronectin and collagens I and III¹²². In addition, the matrix contains aberrant endothelial cells, pericytes, inflammatory cells and macrophages, that secrete chemokines and cytokines, many of which are mitogenic towards both fibroblasts and stellate cells²⁴⁵. The net result is a unique microenvironment in which pancreatic cancer cells thrive and readily invade and metastasise. The abundant connective tissue deposition in PDAC is driven by growth factors such as TGF β and FGF2 within the microenvironment²⁴⁶. These signals in turn activate autocrine and paracrine signalling pathways leading to a tumour growth advantage. Hence the tumour stroma is a vital component in the dynamic process promoting tumour growth and invasion and thus offers a therapeutic target. Since FGF2, a high affinity ligand for FGFR1, is found in abundance in the stroma, it is possible that FGF2 can promote nuclear accumulation of FGFR1 and FGF2, thus explaining the staining pattern I observed in a significant proportion of stromal fibroblasts.

Another possibility for deregulation of FGF signalling in cancer is a result of increased availability of FGFs due to the mobilisation of FGFs from the ECM. Growth factors such as FGF2, TGF β , PDGF and IGF-1 become sequestered in the stroma, which acts as a storage site for these factors²⁴⁷. Invading cancer cells produce MMPs that can act proteolytically to release growth factors that may be sequestered in the ECM⁹⁵. Moreover, cancer cells also shed glypican 1 and syndecan 1, heparan sulfate proteoglycans that can modulate paracrine growth factor signalling by facilitating the interaction between growth factor and their cognate receptor^{96, 97}. Studies have shown that glypican 1 expression is highly upregulated in PDAC stroma and can attenuate the mitogenic response of FGF2²⁴⁸. Together, these alterations result in aberrant epithelial mesenchymal interactions that promote cell proliferation and invasiveness, enhancing tumour spread and

suppressing cancer-directed immune mechanisms²⁴⁷. Thus, the release of growth factors and stimulatory cytokines by invading tumour and stromal cells may provide one explanation for the increase in nuclear FGFR1 and FGF2 found within fibroblasts at the invasive front of PDAC.

CHAPTER IV RESULTS-PART II

Understanding the importance of nuclear FGFR1 and FGF2 in human pancreatic stellate cell behaviour, using 2D cell based assays.

4.1 Introduction

Preliminary results from human PDAC tissue suggest a relationship between nuclear FGFR1 and FGF2 in cancer associated myofibroblasts but not within tumour cells. Furthermore, nuclear FGFR1 and FGF2 may play a role at the invasive front of PDAC. Studies have shown that the vast majority of α SMA positive fibroblasts in PDAC represent activated pancreatic stellate cells (PSCs)^{170, 217}. Thus, the next step in my studies was to examine the expression and localization of FGFR1 and FGF2 and understand the functional effects *in vitro* using immortalised PSCs, together with a panel of PDAC cells. Until this point I haven't discussed in detail the molecular biology of FGFRs, therefore in order to interpret the results, I will first consider and discuss the current understanding of FGF-FGFR cellular signalling.

4.2 Targeting nuclear FGFR1 and FGF2 and the effect on pancreatic stellate cell behaviour

4.2.1 Background

The first FGF was discovered as a mitogen for cultured fibroblasts over three decades ago²⁴⁹. Since then, 22 mammalian FGFs have been identified, with orthologues in many model organisms including *Drosophila*, Nematode and Zebrafish²⁵⁰. The mammalian FGF family comprises 18 secreted FGF ligands,

ranging from 17-34 kDa in size, that signal through four high affinity transmembrane FGF receptors (FGFRs). A fifth receptor, FGFR5, has no tyrosine kinase activity and is thought to negatively regulate signalling by dimerising with FGFRs 1-4 and blocking transphosphorylation²⁵¹. FGFs also bind to heparan sulfate proteoglycans (HSPGs), low affinity receptors that do not transmit a biological signal but function as accessory molecules^{252, 253}.

4.2.2 FGF isoforms and mechanism of action.

An unusual feature of FGF2 and FGF3 is that they are produced as multiple isoforms due to alternative translation start sites^{254 255}. The various isoforms of FGF2 have different sub-cellular localisation and functions and thus, can contribute to different cellular behaviours. Five FGF2 isoforms (18, 22, 22.5, 24 and 34 kDa) have been identified in humans. The various isoforms are generated by alternative initiation of translation of FGF2 mRNA²⁵⁶. Although, as with all *FGF* genes, *FGF2* comprises 3 exons, no splice variants have been detected in mammals. The 18kDa FGF2 (termed low molecular weight, LMW) is translated from a conventional Kozak AUG start codon²⁵⁷ and consists of 155 amino acids, representing the core sequence common to all FGF2 isoforms²⁵⁸. The other isoforms of FGF2 (termed high molecular weight, HMW) are initiated by an in frame upstream CUG site²⁵⁹.

It is generally thought that the HMW forms of FGF2 act within the nucleus and contain a nuclear localisation sequence. However, the 18 kDa LMW form of FGF2 can also travel to the nucleus courtesy of a double arginine motif, Arg116 and Arg118, which is able to facilitate nuclear targeting. Mutation of these amino acids disrupted localisation of both the LMW and HMW of FGF2²⁶⁰. Furthermore, nuclear LMW FGF2 has been shown to stimulate cell growth in low serum concentrations²⁶¹.

However the target genes of LMW and HMW FGF2 in the nucleus may be different and, therefore, theoretically capable of eliciting different biological responses²⁶².

Most FGFs are secreted. However, it is still not fully understood why FGF1, FGF2 and FGF9, which lack leader sequences for secretion, are released from the cell. The lack of signal peptide in these ligands implies that they are transported to the extracellular space by an alternative mechanism to the classical polypeptide secretion pathway, dependent on the ER and Golgi apparatus²⁵⁵. A number of hypotheses have been proposed as to how these ligands are released, including mechanical damage²⁶³. However this theory does not explain why biologically active FGF2 is released during development²⁵⁵. It is clear further studies are required to fully understand how FGF1, FGF2 and FGF9 are able to exert their extracellular actions.

4.2.3 FGFR activation

FGFRs are comprised of an extracellular ligand-binding domain linked to an intracellular catalytic protein kinase core, via a single pass transmembrane domain. Normally, the extracellular ligand binding domain of the receptor consists of three immunoglobulin (Ig) domains (designated D1-3,) a stretch of seven to eight acidic residues in the region connecting D1 to D2 (designated the 'acid box') and a conserved positively charged region, in D2, that binds heparin²⁶⁴.

A feature unique to the FGFR family of RTKs is the variety of isoforms that are generated by alternative splicing of the FGFR mRNAs²⁶⁵. Many splice isoforms have been described, the principal ones being alternative splicing of the D3 domain of FGFR1-3, which determines the sequence of the carboxy-terminal half of the third, membrane-proximal, Ig domain and strongly dictates ligand-receptor binding specificity²⁵⁰ (Table 4. 1). The first half of the D3 domain is encoded by exon IIIa, which is spliced to either exon IIIb or IIIc, both of which splice to the exon encoding the transmembrane (TM) region. Other splicing events lead to inclusion or exclusion of the most N-terminal Ig loop, which is thought to act in an autoregulatory manner²⁶⁶, or generation of a secreted receptor when exon IIIa splices directly to the transmembrane region, generating a premature termination codon (Figure 4.1)²⁶⁷.

In general, FGFRb isoforms are expressed on epithelial cells, while FGFRc isoforms are restricted to mesenchymal cell types, this being particularly true for FGFR2 and FGFR3¹⁷⁹. This lineage specific expression of the FGFR 'b' and 'c' isoforms allows establishment of paracrine signalling loops between epithelial and mesenchymal tissues during development.

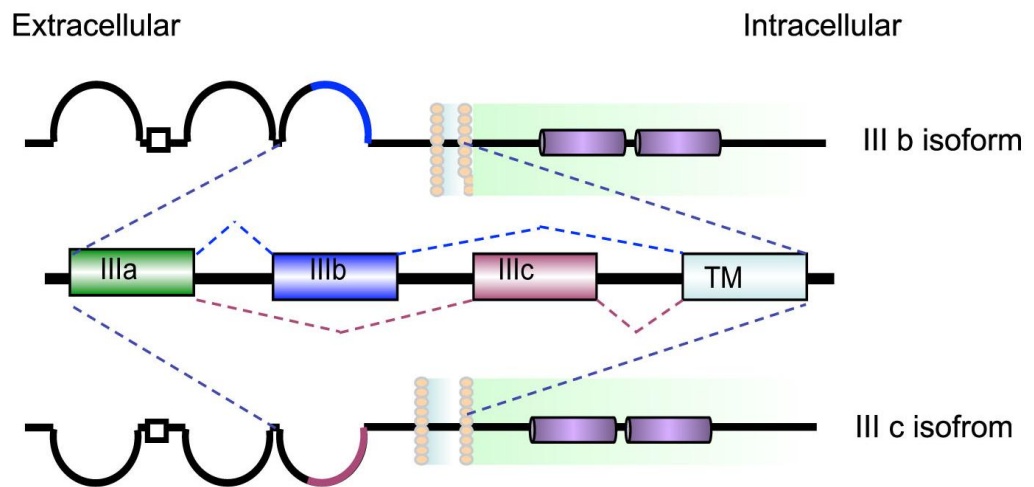


Figure 4. 1 Alternative splicing of FGFR.

Ligand binding specificity is generated by alternative splicing of the Ig III domain. The first half of Ig III is encoded by an invariant exon (IIIa), which is spliced to either exon IIIb or IIIc, both of which splice to the exon that encodes the transmembrane (TM) region²⁵⁰.

FGF subfamily	FGF	FGFR specificity
FGF1	FGF1 FGF2	All FGFRs FGFR1c, FGFR3c > FGFR2c, FGFR1b, FGFR4
FGF4	FGF4 FGF5 FGF6	FGFR1c, FGFR2c > FGFR3c, FGFR4
FGF7	FGF3 FGF7 FGF10 FGF22	FGFR2b > FGFR1b
FGF8	FGF8 FGF17 FGF18	FGFR3c > FGFR4 > FGFR2c > FGFR1c >> FGFR3b
FGF9	FGF9 FGF16 FGF20	FGFR3c > FGFR2c > FGFR1c , FGFR3b >> FGFR4
FGF19	FGF19 FGF21 FGF23	FGFR1c, FGFR2c, FGFR3c, FGFR4 (weak activity)
FGF11	FGF11 FGF12 FGF13 FGF14	No known activity

Table 4. 1 Specificity of ligand for FGFR isoforms

FGFR1-3 are alternatively spliced, while FGFR4 is not. This alternative splicing event is regulated in a tissue specific manner and dramatically affects ligand binding. For example epithelial expressed FGFR2b can be activated by mesenchymal FGF7 and FGF10, however these ligands show no activity towards mesenchymally expressed FGFR2c²⁶⁸.

Classically, FGFRs are activated when two FGF molecules (in concert with HSPG) bind to the IgII and IgIII extracellular domains, facilitating receptor dimerisation²⁶⁴. The crystal structure of FGF in complex with the ligand binding domain of FGFR has given insight into the determinants that govern FGF:FGFR specificity and binding. Crystal structures show a 2:2 FGF:FGFR complex in which FGF interacts with IgII and IgIII and with the linker that connects these two domains within one receptor. This complex is stabilised by a secondary binding site involving interactions between FGF and the IgII domain of the second receptor as well as receptor: receptor interactions. FGF:FGFR interactions under normal physiological conditions are not sufficient to stabilise FGFR dimers. HPSG binding across the two IgII domains and adjoining the two FGF molecules is essential for dimerisation of the FGF:FGFR complex^{264, 269}.

4.2.4 Downstream signalling

Ligand induced dimerisation of FGFRs leads to a conformational shift in the receptor structure and release of kinase auto-inhibition. This results in a 50-100 fold increase in kinase activity of the receptor, resulting in activation through trans-phosphorylation of several tyrosine residues within the intracellular domain^{270, 271}. This phosphorylation increases the receptor kinase activity, generating docking sites for downstream signalling molecules and consequent activation of multiple signal transduction pathways. FGFR activation leads to phosphorylation of a number of intracellular proteins such as FGFR substrate 2 (FRS2) and Phospholipase Cy (PLC γ)^{272, 273} (Figure 4. 2). Binding of PLC γ at the activated tyrosine at the C terminus of the receptor results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into two secondary messengers; diacylglycerol and inositol 1, 4, 5-triphosphate. This in turn leads to release of intracellular calcium stores and

activation of the serine-threonine kinase, protein kinase C (PKC)²⁷⁴. Activated FRS2, at the juxtamembrane domain of the receptor, serves as an adaptor molecule for a number of signalling complexes that promote activation of RAS/mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3 kinase)/Akt signalling pathways²⁷¹. In addition, src homology 2 domain containing transforming protein B (shb), Src kinase, ribosomal S6 protein kinase (RSK), signal transducers and activation of transcription (STATs) and Crk have all been shown to be involved in transducing FGF mediated signalling²⁷⁵.

The predominant signalling pathway activated downstream of FGFRs in development is the MAPK signal transduction pathway²⁷⁶. Although, in most cellular contexts, FGFs will induce cellular proliferation and migration, in certain context FGFs may induce cell-cycle arrest²⁷⁷. FGF signalling can profoundly affect a number of basic cellular processes. How each process is affected varies according to cell type and maturation stage, the nature of the ligand, the nature of the receptor, and utilisation of distinct signalling pathways, roles of downstream effectors in different cell types and differential regulation of gene transcription.

Several mechanisms exist to limit FGFR activation, FRS2 can form an FGFR inhibitory complex through the recruitment of Cbl, which leads to proteosomal targeted degradation of FGFRs. Similarly, signalling by FGFRs (and other RTKs) can induce the transcription and activity of a group of MAPK inhibitors such as Sprouty (SPRY), Sprouty related proteins (Spred1,2) MAPK phosphatase/dual specificity phosphatase (Mkp/Dusp) family members and 'similar expression to FGF' (SEF) proteins that modulate receptor signalling at several points in the FGFR induced signal transduction cascade^{278, 279}. Similarly, the first Ig domain of the

receptor may interact with the ligand binding domain of IgII and IgIII and thus interrupt FGF binding to the receptor. This autoinhibition would prevent activation of FGFRs by abundant HSPGs in the ECM or cell surface. Thus the extracellular domain of the FGFR can have a role in either an activation or inhibition of cell growth dependent on the context.²⁸⁰.

Alternatively, context-specific FGF signalling can be modulated by activation or repression of other signalling pathways. In particular FGFs and Wnts have been shown to interact in a variety of developmental systems and, together, elicit cellular responses that are distinct from the effects of each individual factor ²⁷⁰. Finally, evidence shows that differences in signal potentiation between FGFRs may also affect context specific FGF signalling. Although the network of pathways through which FGFRs signal are similar, it has been shown that signalling through FGFR1 drives stronger downstream signal activation than through FGFR4 ²⁸¹.

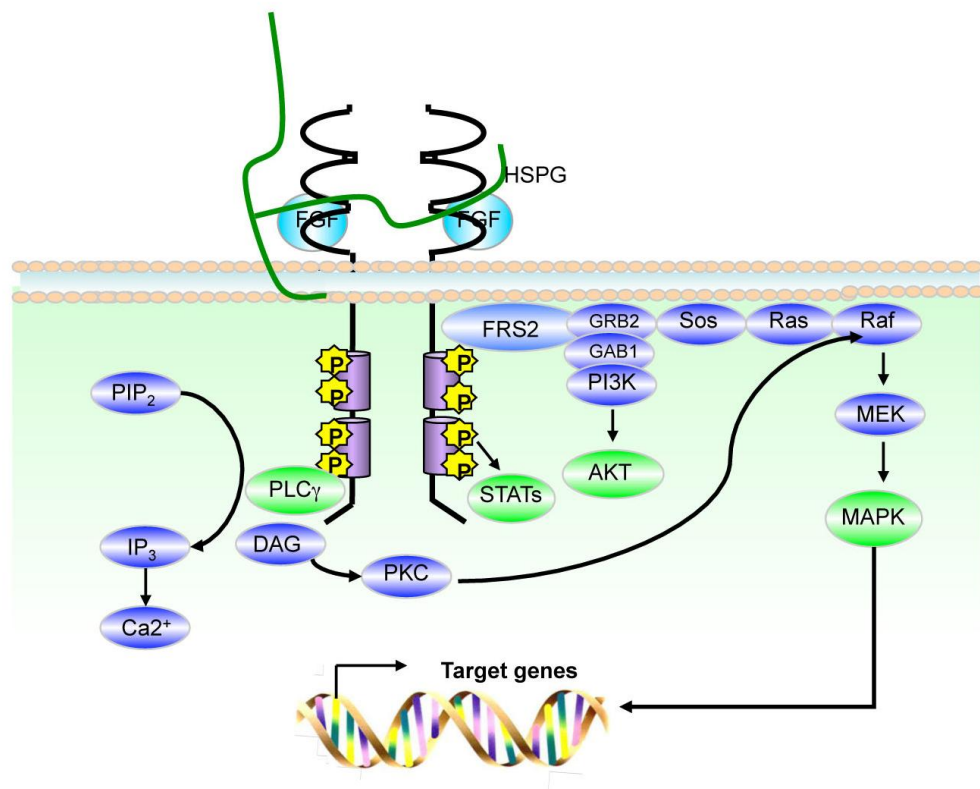


Figure 4. 2 FGFR signalling pathways

Following ligand binding and receptor dimerisation, the kinase domains undergo trans-phosphorylation, leading to the docking of adaptor proteins and the activation of four key downstream pathways, MAPK, PI3K, signal and transducer and activator of transcription (STAT) and phospholipase C γ (PLC γ)²⁸²

The different FGFRs play an important role in both embryogenesis and the adult organism and embryological studies investigating the effects of loss of FGFRs demonstrated their importance for organ development, including that of the pancreas²⁸³. During development, FGFR signalling orchestrates a plethora of processes²⁷⁵. FGF signalling is key to mesenchymal-epithelial interaction and FGFRs are well known inducers of mesoderm. In particular, endodermal to mesenchymal FGF signalling plays an important role in pancreatic development. FGF4 serves to delineate the developing pancreatic mesenchyme early in development and without this molecular signal to the mesenchyme, the proper formation of the endoderm derived pancreas is inhibited^{34, 35}. Later in development, FGF4 can induce the mesenchyme to persist, and enhance exocrine differentiation of the adjacent epithelium³⁶.

In addition to FGF2 signalling from the notochord, which plays a critical role in maintaining dorsal pancreatic development^{45, 284}, FGFs 1, 4, 7 and 10 are all expressed by the pancreatic mesenchyme during development and play a vital role in pancreatic organogenesis³⁷. Most of these ligands are able to bind to the dominant FGFR expressed in the pancreatic epithelium, FGFR2b³⁸. FGFR2b appears to be the key receptor for pancreatic development and may be implicated in the activation and proliferation of pancreatic progenitor cells⁴⁰, promoting pancreatic epithelial proliferation, without eliciting cellular differentiation^{37, 40, 41}. Mouse embryos that lack FGFR2b display altered pancreatic development, due to a lack of early proliferation and branching morphogenesis of the pancreas³⁸. Later in development, these FGF ligands expressed by the mesenchyme promote cells to differentiate to an exocrine lineage and inhibit endocrine progenitor proliferation^{36, 42}. Finally, FGF signalling from the mesenchyme to the epithelium indirectly activates Notch signalling. Several studies have shown that Notch signalling is critical for cell fate

determination within the pancreas⁴⁷⁻⁴⁹. Mouse models have shown that Notch is activated by FGFR2b paracrine signalling, resulting in an increase in expression of the Notch effector molecule hairy/enhancer of split (Hes1)³⁵. After the appearance of mature pancreatic buds, FGF and Notch signalling reduces and initiation of acinar differentiation occurs²⁸⁵.

In addition to the importance of FGF/FGFRs for pancreas development, FGFR signalling is important for formation of the nervous system, the limbs, the midbrain and the lungs²⁵⁵. FGF signalling also plays an important role in the development of the embryonic mammary gland²⁸⁶. In the adult, FGFR signalling regulates tissue repair, angiogenesis and inflammation²⁵⁵. Thus, given the role of FGF signalling in the developing embryo and the adult, it is not surprising that this pathway is often hijacked by many cancer cells including PDAC²⁶⁵. As such several FGFR tyrosine kinase inhibitors and FGFR blocking antibodies are under development by pharmaceutical companies and many are in early clinical trials²⁸⁷.

4.2.5 FGF signalling in PDAC

FGF1, FGF2, FGF5, FGF7 and FGF10 have all been shown to be overexpressed to various degrees in PDAC, and over expression of FGF2 has been associated with shorter post-operative patient survival^{77, 179-181, 288, 289}. A role for FGF1 and FGF2 signalling in modulation of epithelial-to-mesenchymal transition (EMT) and motility has also been proposed²⁹⁰. One explanation for the over-expression of FGFs in PDAC is the frequent expression of other growth factors and cytokines that may drive the upregulation of these ligands in PDAC. Indeed, *in vitro* studies have shown that expression of FGF-5 and FGF-7 mRNAs is upregulated in cultured fibroblasts, upon stimulation with tumour necrosis factor alpha (TNF- α), EGF and platelet-

derived growth factor (PDGF), as well as interleukin 1- β . PDAC cells also over express the 'c' variant of fibroblast growth factor receptor 1 (FGFR1) which is normally expressed in mesenchymal cells¹⁸¹.

The presence of the 'c' isoform of FGFR1 in PDAC cells of epithelial origin raises the possibility that malignant transformation in PDAC leads to de-differentiation and subsequent aberrant expression of the mesenchymal receptor isoform. Indeed, switching from FGFR2b to FGFR2c expression in prostate cancer cells has been associated with an increase in invasion, leading to a stromal independent, undifferentiated tumour type²⁹¹. Furthermore, down-regulation of FGFR2b in keratinocytes resulted in malignant transformation, whereas re-expression of FGFR2b resulted in growth inhibition and induction of differentiation in prostate, bladder and human salivary adenocarcinoma cells²⁹²⁻²⁹⁴. Thus, exon-switching from b to c isoform in PDAC may be important for driving a more malignant phenotype. In addition, PDAC cells have been shown to express the 2-lg form of FGFR1, while normal pancreas predominantly expresses the full-length 3-lg receptor²⁸⁹. This raises the possibility of aberrant FGFR1 splicing in PDAC, with the ductal cells overexpressing the 2-lg isoform. Indeed, overexpression of the 2-lg splice form of FGFR1 occurs in astrocytomas, prostate and breast cancer, whereas the corresponding 3-lg form predominates in normal breast tissue²⁹⁵. Consistent with a role for FGF signalling in supporting PDAC growth, a dominant negative FGFR1 mutant or RNAi to glypican-1 (a membrane heparin sulphate proteoglycan that facilitates FGF-FGFR interactions) can inhibit growth of PDAC cell lines *in vitro* and suppress tumourigenic potential in mouse xenografts^{179, 248, 296, 297}.

Moreover, FGF 10 signalling through the b isoform of FGFR2 has been shown to increase PDAC cell migration and invasion and is associated with increased expression of membrane type 1-matrix metalloproteinase (MT1-MMP) and TGF β 1 mRNA²⁸⁹. However, given that FGF 10 also activates FGFR1b, and that expression of this isoform was not explored in this study, it is difficult to conclude that the metastatic phenotype of these cells was exclusive to FGFR2b signalling. Further studies are required to fully understand how FGF ligands, which activate different receptor isoforms on cancer cells, are orchestrated to induce a malignant phenotype in PDAC.

4.2.6 Nuclear FGFRs

As discussed above, FGFs have been shown to have nuclear functions as well as their role as secreted glycoproteins. There is now mounting evidence that FGFRs can also traffic to the nucleus, where they may carry out their function in a different manner to that of the classic RTK signalling pathway²⁹⁸. Following activation at the cell surface, both ligand and receptor are internalised and, in some instances, FGFR/FGF complexes can be translocated to the nucleus, where they regulate cell proliferation (however, the source of nuclear FGFR is still under debate)^{128, 129}. Much of the research that has been carried out on nuclear FGFRs has been focused on FGFR1. However, both FGFR2 and FGFR3 have been shown to translocate to the nucleus. The role of nuclear FGFR3 is not understood but has been shown in the nucleus of malignant and non-malignant epithelial breast tissue²²⁷, whereas nuclear FGFR2 has been identified in sertoli cell precursors in the testes, where it may play a role in regulating sertoli cell differentiation and testis development²⁹⁹.

In neuronal cells, nuclear FGFR1 (full length) has been shown to colocalise at nuclear speckle-like domains, which correspond to sites of RNA Pol II mediated transcription and co-transcriptional pre-mRNA processing, located at the edge and centre of the speckles, respectively³⁰⁰. This suggests that nuclear FGFR1 may play a role in regulating gene expression. Indeed, chromatin immunoprecipitation (ChIP) studies have shown that nuclear FGFR1, together with CREB binding protein (CBP), a common transcriptional co-activator, as well as other DNA binding proteins, is able to bind to the promoters of the genes encoding the dopamine synthesis enzyme *tyrosine hydroxylase (TH)* and *FGF2*, regulating neuronal differentiation and proliferation^{226, 300-308}. In addition, our laboratory has recently shown that a cleaved intracellular form of FGFR1 localises to the nucleus in metastatic breast cancer cells, where the receptor can bind to genes which regulate breast cancer cell migration and invasion³⁰⁹. This mechanism provides a novel mechanism in which nuclear FGFR1 may regulate breast cancer cell behaviour. Whether nuclear FGFR1 may play a role in regulating gene transcription in other cell types is yet to be determined. Given that FGFR1 and FGF2 localised to the nucleus in α SMA positive myofibroblasts in PDAC tissue, and corresponded with fibroblasts at the invasive front, the question arises whether FGFR1 and FGF2 may regulate stellate cell behaviour in PDAC and thus may offer a potential therapeutic target.

4.2.7 In vitro FGFR1 and FGF2 expression and localisation in human pancreatic cancer cells and stellate cells

As the first step in elucidating the role of nuclear FGFR1 and FGF2, I investigated the localisation and expression of FGFR1 and FGF2 by screening a panel of poorly- (Figure 4.3) and well-differentiated PDAC cell lines (Figure 4. 4), normal pancreatic ductal epithelial cell lines (HPDE³¹⁰ and DEC-hTERT¹⁹⁹) and immortalised (PS1¹⁷⁰) (Figure 4. 5) and primary stellate cells (Figure 4. 6) (by immunostaining and Western blot analysis). FGFR1 immunostaining frequently revealed a low level of plasma membrane staining; a result of the technique used to permeabilise the cells. Cytoplasmic localisation of FGFR1 has been described previously³¹¹. Many well-differentiated cancer cell lines (Capan-1, Capan-2, CFPAC-1, 818.1) exhibited mainly cytoplasmic or peri-nuclear localisation of FGFR1. However, in PS1 and some poorly-differentiated cancer cell lines (AsPc-1, MIA PaCa-2, PANC-1, PaTu8898S, SUIT-2,), FGFR1 was strongly nuclear and exhibited a specked distribution. Western blotting revealed that FGFR1 expression (full length ~ 160 kDa) was stronger in many of the poorly-differentiated cancer cells lines (Figure 4. 5) than normal ductal epithelial or well-differentiated PDAC cell lines. The MCF7 breast cancer cell line was used as a positive control as this cell line has been shown previously to express full length FGFR1³⁰⁹.

Immunostaining for FGF2 showed cytoplasmic or peri-nuclear staining in cancer and normal ductal epithelial cell lines. In contrast, FGF2 was localised predominantly within the nuclei (nucleolar and diffuse nuclear staining) in PS1 cells. Western blotting revealed two distinct bands for FGF2, which correspond to HMW (~24 kDa) and LMW (~18 kDa) forms of the protein. Stellate cells showed strong expression of

both HMW and LMW FGF2 isoforms, as did many poorly-differentiated cancer cell lines. However, FGF2 expression was weak in many well-differentiated cancer cell lines and several lines showed no FGF2 expression (Figure 4. 5, 818.1 and CFPAC-1 and Capan-2).

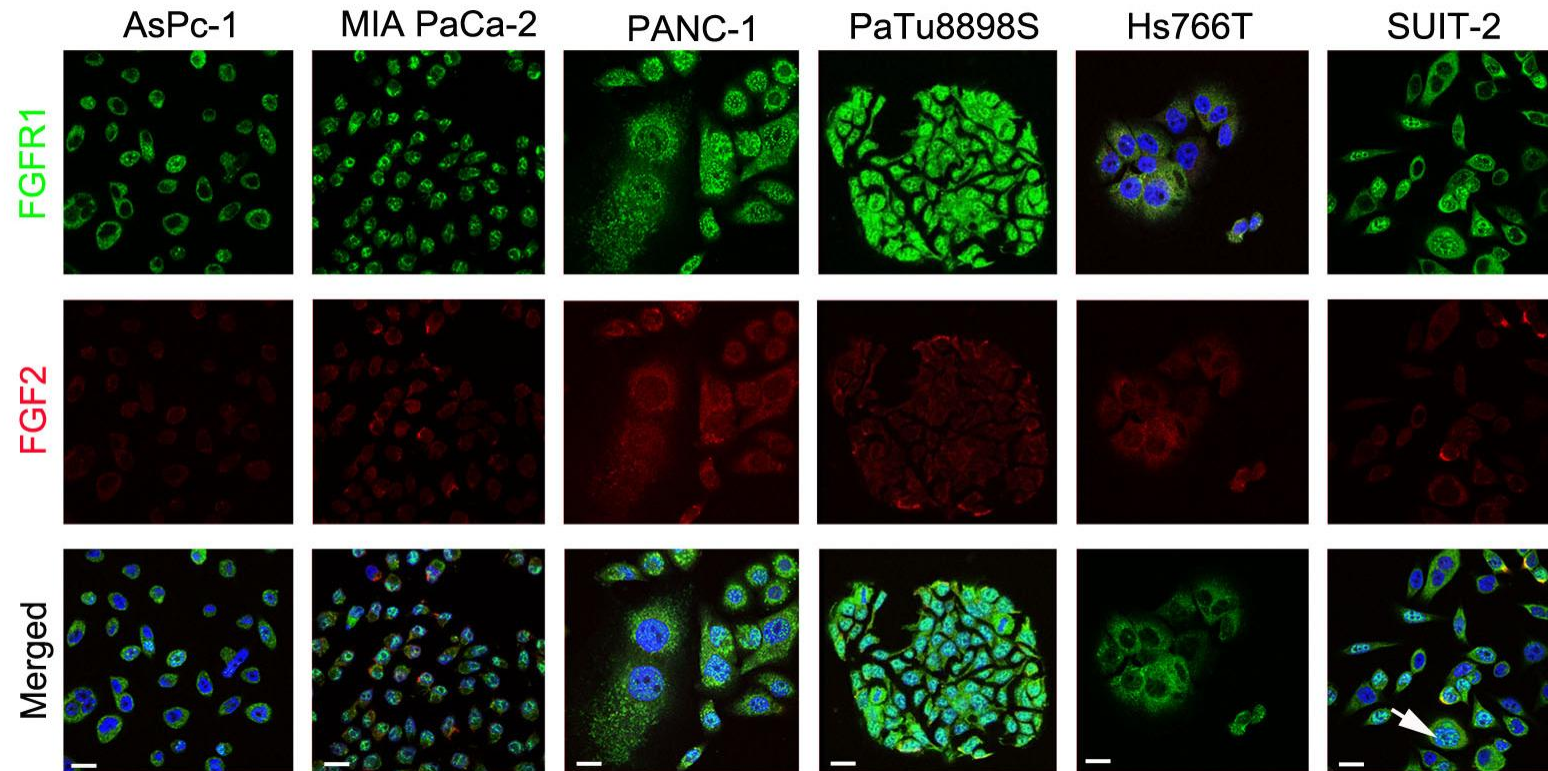


Figure 4. 3 FGFR1 and FGF2 expression in poorly differentiated pancreatic cancer lines.

Coverslip-plated cells were stained with antibodies to FGFR1 (green) or FGF2 (red), nuclei were counter stained with DAPI (blue). Poorly-differentiated (AsPc-1, MIA PaCa-2, PANC-1, PaTu8898S, Hs766T, SUIT-2) pancreatic cancer cell lines showed cytoplasmic or peri-nuclear FGF2 staining. Most of the poorly-differentiated cells lines showed strong nuclear FGFR1 staining also (arrow) Scale Bar: 20 μ m.

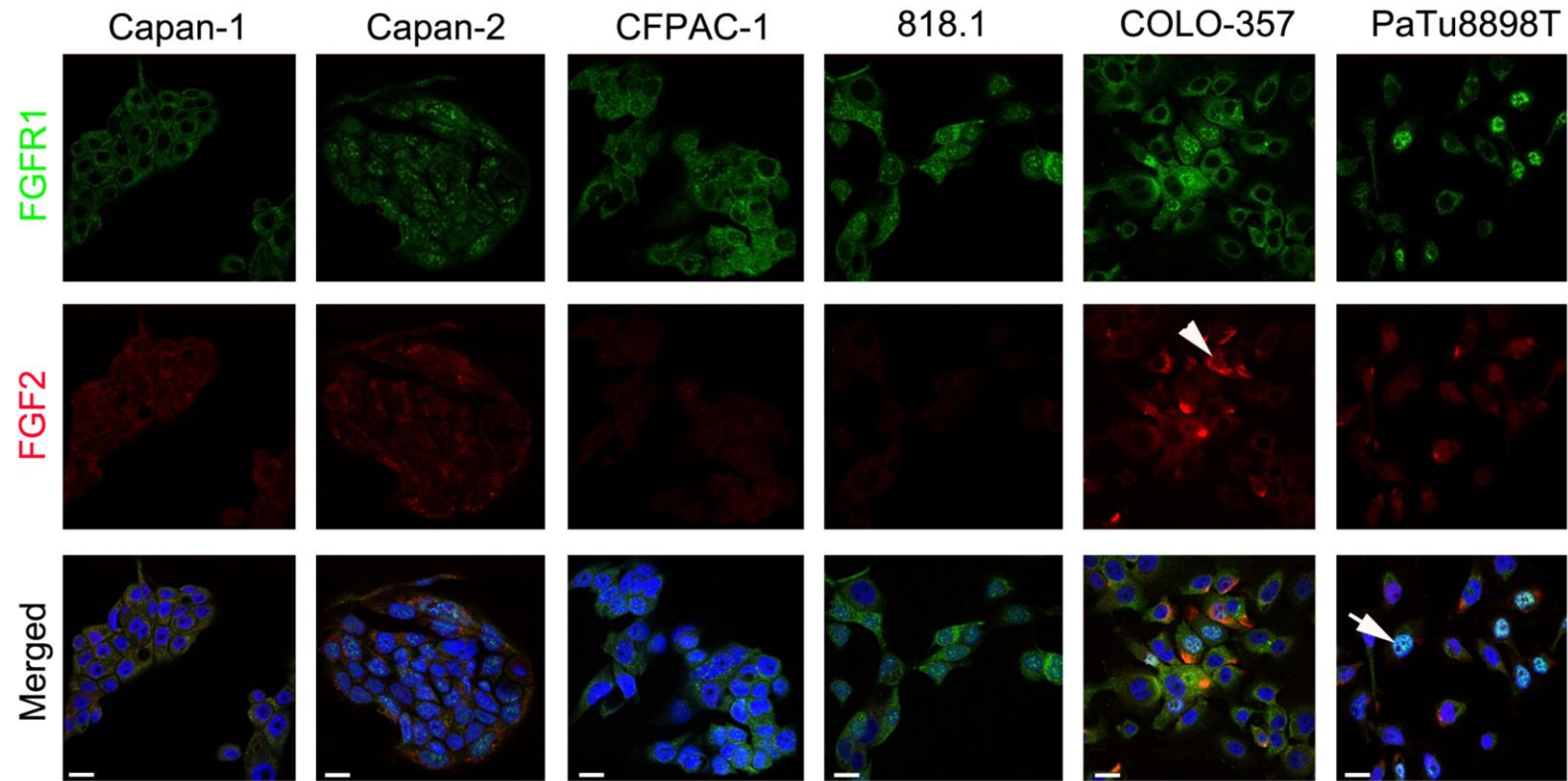


Figure 4. 4 FGFR1 and FGF2 expression in well differentiated pancreatic cancer lines.

Coverslip-plated cells were stained with antibodies to FGFR1 (green) or FGF2 (red), nuclei were counter stained with DAPI (blue). well-differentiated (B: Capan-1, Capan-2, CFPAC-1, 818.1, COLO-357, PaTu8898T) pancreatic cancer cell lines showed cytoplasmic or peri-nuclear FGF2 staining (arrow head). FGFR1 was mainly cytoplasmic but nuclear in PaTu8898T (arrow) and COLO-357 cells. Scale Bar: 20 μ m

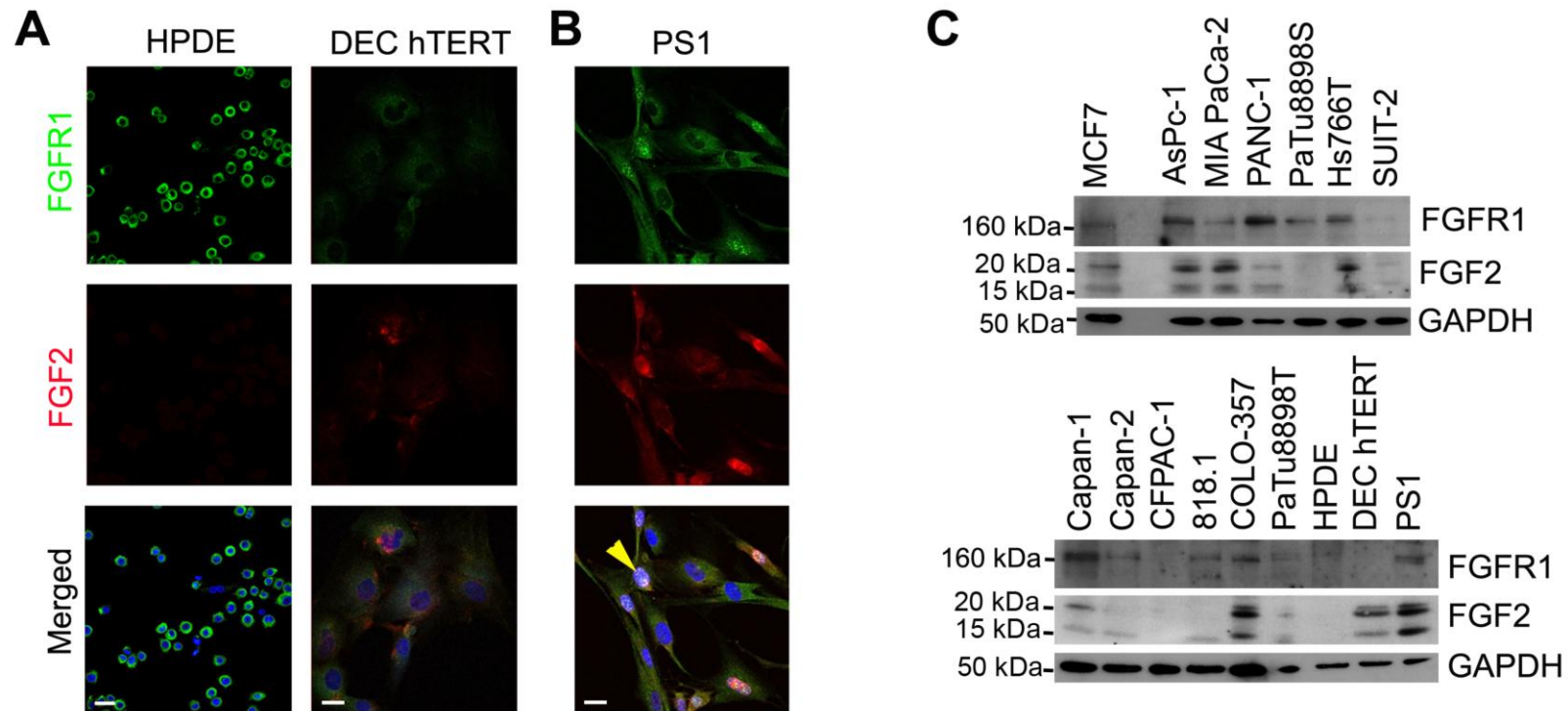


Figure 4. 5 FGFR1 and FGF2 expression in normal ductal and stellate cell lines.

Coverslip-plated cells were stained with antibodies to FGFR1 (green) or FGF2 (red), nuclei were counter stained with DAPI (blue). FGFR1 and FGF2 were absent from the nuclei of control normal epithelial cell lines HPDE and DEC-hTERT (A). Stellate cells (PS1, B) showed strong nuclear FGF2 and FGFR1 (yellow arrow head). Immunoblot analyses (C) were performed with lysates from poorly- and well-differentiated, normal ductal epithelial (HPDE and DEC-hTERT), pancreatic stellate (PS1) cell lines. MCF-7 breast cancer cell line was used as a positive control. Tubulin was used as a loading control. Pancreatic cancer cell lines showed moderate expression of high molecular weight (HMW, 24 kDa) and low molecular weight (LMW, 18 kDa) forms of FGF2, however the majority of well-differentiated cell lines showed no expression of either isoform of FGF2. Weak expression of FGF2 was detected in control cell lines HPDE and DEC-hTERT. PS1 cells showed strong expression of both HMW (24 kDa) and LMW (18 kDa) forms of FGF2. Whilst FGFR1 expression was weak in normal epithelial cell lines, its expression appeared higher in many poorly-differentiated cell lines. Scale Bar: 20 μ m.

4.2.8 FGF2 and FGFR1 colocalise in the nucleus of pancreatic stellate cells

The observed pattern of immunostaining in PSCs could be due to peri-nuclear, nuclear membrane or intranuclear staining. Therefore, I used confocal z-stack analysis to distinguish between these locations. Analysis of FGFR1 and FGF2 in PS1 cells revealed that FGF2 staining was only observed in those stellate cells in which FGFR1 was nuclear, and co-localised at distinct speckles within the nuclear interior (Figure 4.7, shown in yellow). Nuclear FGFR1 and FGF2 co-localised in around 40% of stellate cells, which was a similar distribution to myofibroblasts found in PDAC tissue (Figure 3. 1). I was able to confirm this observation by isolating cancer associated primary pancreatic stellate cells (in an ethically approved manner LREC 07/H0707/87) using the outgrowth method²⁰⁰. Immunostaining for FGFR1 and FGF2 in primary stellate cells confirmed the nuclear localisation of FGF2 and FGFR1, which I observed in the PS1 cell line (Figure 4. 6)

FGF2 is a potent mitogen often secreted by many cell types²⁵⁵. Immunostaining of sections from PDAC has shown that increased expression of cytokines such as IGF and FGF2 maybe secreted by mesenchymal cells^{77, 312}. FGF2 secreted by fibroblasts can induce proliferation and transformation of epithelia at the tumour-stroma interface²⁴⁶. Furthermore, FGF2 can activate stellate cells and this activation can be inhibited by the presence of neutralising antibodies against FGF2¹²². To asses if the PS1 cell line was capable of secreting FGF2, I performed Western blot analysis for FGF2 expression using conditioned media taken from PS1 and PDAC cancer cell lines, MiaPaCa2 and COLO-357 (poorly- and well-differentiated PDAC cancer cell lines, respectively, both of which express FGF2). Stellate cells, but not cancer cells, secreted both HMW and LMW FGF2 (Figure 4.7). Given the close

proximity of stellate cells to cancer cells *in vivo*, this may be one way in which stellate cells can induce cancer cell activation in a paracrine fashion. Similarly, FGF2 secreted by stellate cells may lead to auto-activation via an autocrine loop.

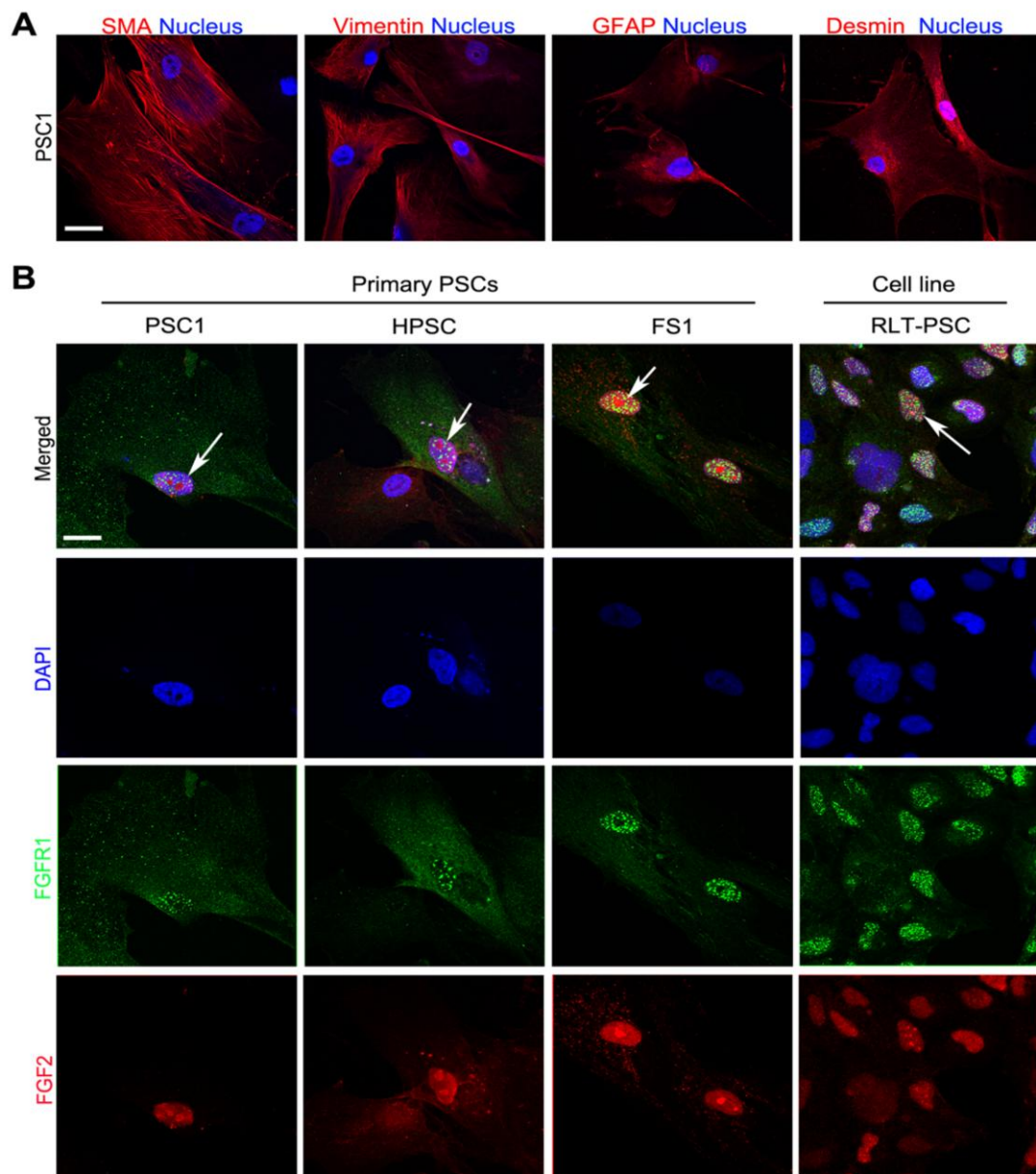


Figure 4. 6 FGFR1 and FGF2 expression in primary pancreatic stellate cells.

Pancreatic stellate cells (PSCs) isolated from resected human cancer specimens (Methods) were characterised by expression of α SMA, GFAP, desmin and vimentin markers (A). (B) Primary pancreatic stellate cells (PSC1, HPSC, FS1) as well as a pancreatic stellate cell line (RLT-PSC) demonstrated speckled nuclear localisation of FGFR1 (arrow) as well as diffuse nuclear FGF2. Scale Bar: 20 μ m.

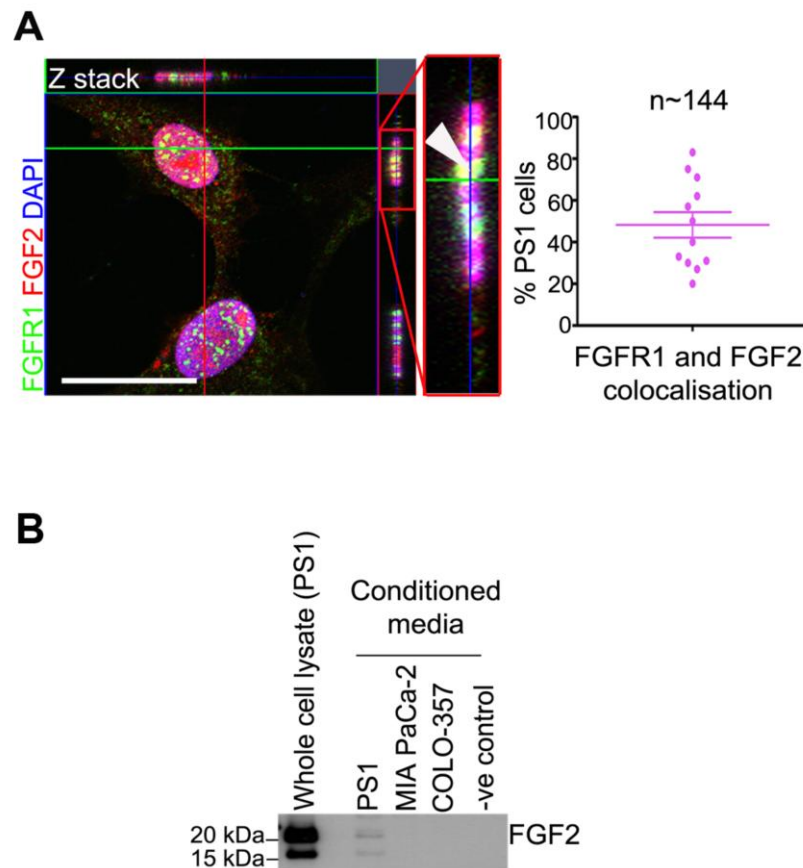


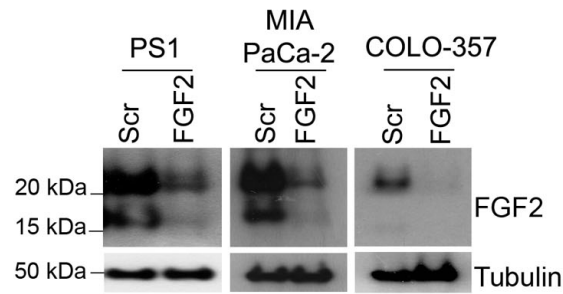
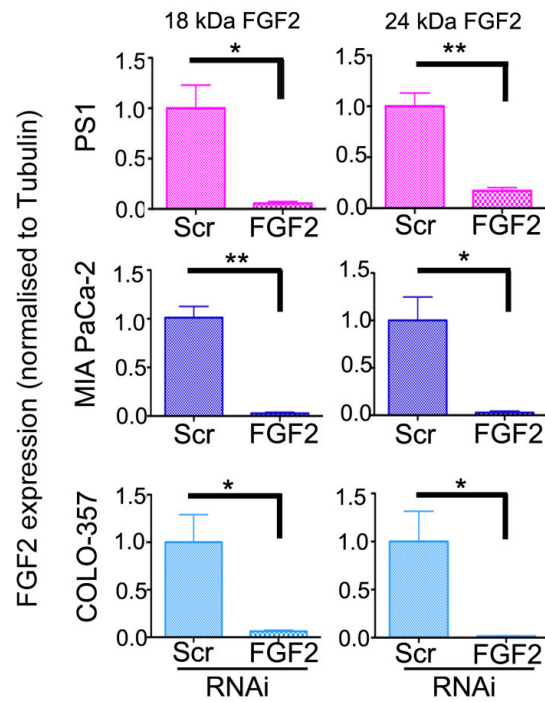
Figure 4. 7 Nuclear FGFR1 and FGF2 colocalise in PSCs

(A) Pancreatic stellate cells (PS1 cell line) showed punctate, nuclear speckles of FGFR1 staining (green) and diffuse nuclear FGF2 (red), with colocalisation (yellow, indicated by arrow head) confirmed by optical sectioning through the Z-axis (Z-stack) and pixel colocalisation techniques (50% of PS1 cells). Nuclear FGF2 was apparent only in those cells with nuclear FGFR1. (B) Serum-free conditioned media confirmed PS1, but not cancer cells, secrete high- and low-molecular weight (HMW 24 kDa and LMW 18 kDa, respectively) forms of FGF2. Whole cell lysate (untreated PS1 cells) and serum-free medium were used as positive and negative controls respectively. Scale Bar, 20 μ m.

4.2.9 Relationship between nuclear FGFR1 and FGF2 in pancreatic stellate cells.

The observation that FGFR1 and FGF2 colocalise in the nucleus of stellate cells suggests that there may be a reciprocal relationship. In glial cells, FGFR1 and FGF2 have shown to localise to the nucleus, where the presence of FGFR1 may mediate the proliferative effects of FGF2²²⁶. Therefore, to assess the relationship between nuclear FGF2 and FGFR1 in stellate and cancer cells, PS1, Mia PaCa-2 and COLO-357 cell lines were subjected to RNAi mediated knock-down of FGF2. Treatment of PS1 cells with FGF2 RNAi for 72 hours resulted in an efficient and reproducible knock-down of both isoforms of FGF2 protein, as shown by Western blot and also by confocal microscopy (Figure 4. 8). Abolition of FGF2 in PS1 cells had a profound effect on nuclear FGFR1, as quantified using confocal microscopy (Figure 4. 9).

To confirm the sub-cellular distribution of nuclear FGFR1 and FGF2, I isolated nuclear and cytoplasmic fractions. The purity of the nuclear and cytoplasmic fractions was confirmed by using markers specific for these sub-cellular compartments (Lamin A/C and Tubulin, respectively). Sub-cellular fractionation confirmed that FGF2 (HMW and LMW) and full length FGFR1 localised to the nucleus in PS1 cells, and that nuclear FGFR1 was dependent on nuclear FGF2 (Figure 4. 9). In contrast, using confocal microscopy and sub-cellular fractionation, I was able to demonstrate that knock-down of FGF2 had no effect on nuclear FGFR1 in PDAC cells (Figure 4.10). Importantly, I could demonstrate this relationship between nuclear FGFR1 and FGF2 in PS1 cells by abolishing FGFR1 and observing significant reduction of nuclear FGF2 (Figure 4.11).

A**B****Figure 4. 8 RNAi mediated knock-down of FGF2**

(A) Cell lines (PS1, MIA PaCa-2 and COLO-357) were transfected with either FGF2 siRNA (FGF2) or scrambled non-targeting (Scr) siRNA for 72 hours. FGF2 expression levels were analysed by immunoblotting. Tubulin was used as a loading control. (B) The ratio of FGF2 (HMW, 24kDa and LMW) to Tubulin was determined by densitometric analysis. FGF2 RNAi treatment achieved >90% knock-down of FGF2 protein levels in PS1, MIA- PaCa-2 and COLO-357 cells. *P < 0.05, **P < 0.01, Student's t-test. Data summary represented by mean \pm SEM. Images are representative of at least three independent experiments.

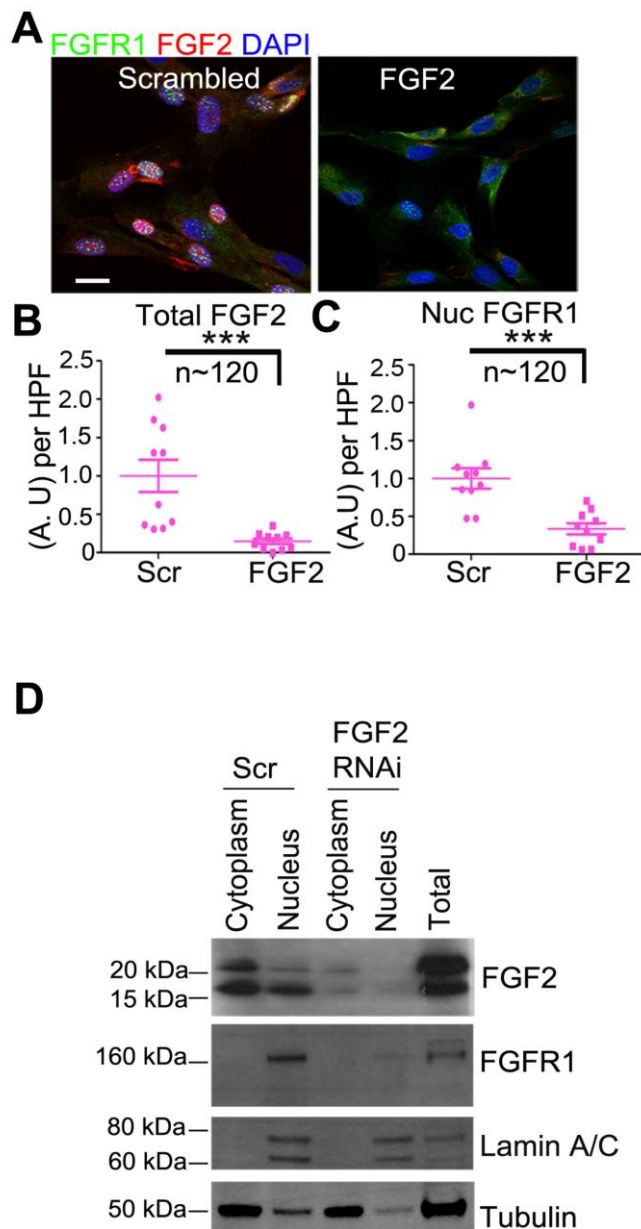


Figure 4. 9 Relationship between nuclear FGFR1 and FGF2 in stellate cells

(A-C) RNAi mediated knock-down of FGF2 resulted in a significant reduction in nuclear FGFR1 in PS1 cells in comparison to scrambled RNAi (Scr), as demonstrated by microscopic analysis. (D) Sub-cellular fractionation and subsequent immuno-blotting confirmed that FGFR1 expression was dependent upon FGF2. Total lysate was used as a positive control. Lamin A/C and tubulin were used as markers of fraction purity and loading controls. Scale Bar, 20 μ m. *** $P < 0.001$. Student's t-test. Data summary represented by mean \pm SEM. For analysis of nuclear FGFR and FGF2, each data point shown represents an average of total or nuclear FGFR or FGF2 per field. Several fields were counted per experiment. The total number of PS1 cells analysed is recorded in the figure (n). For all data, images are representative of three independent experiments.

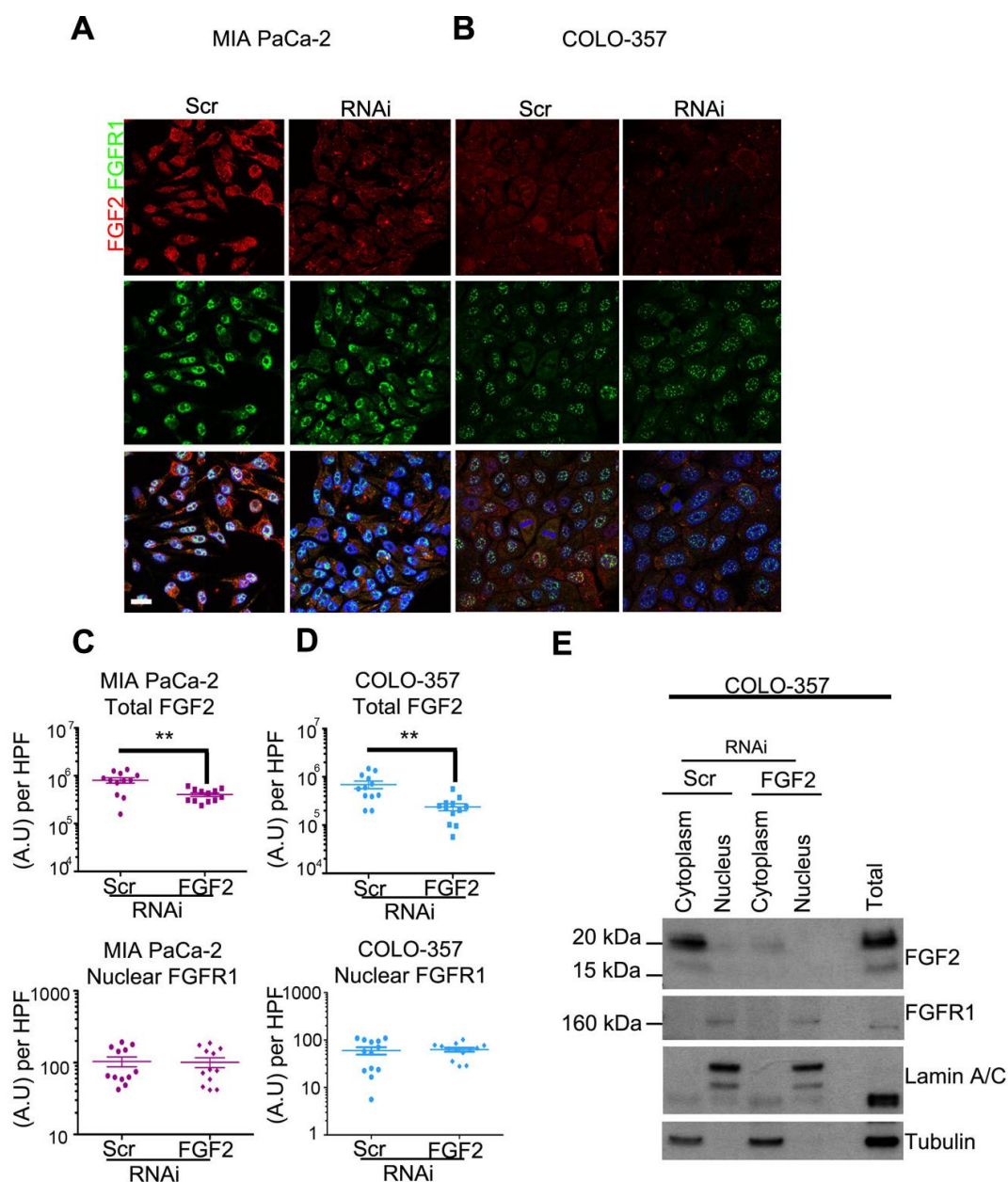
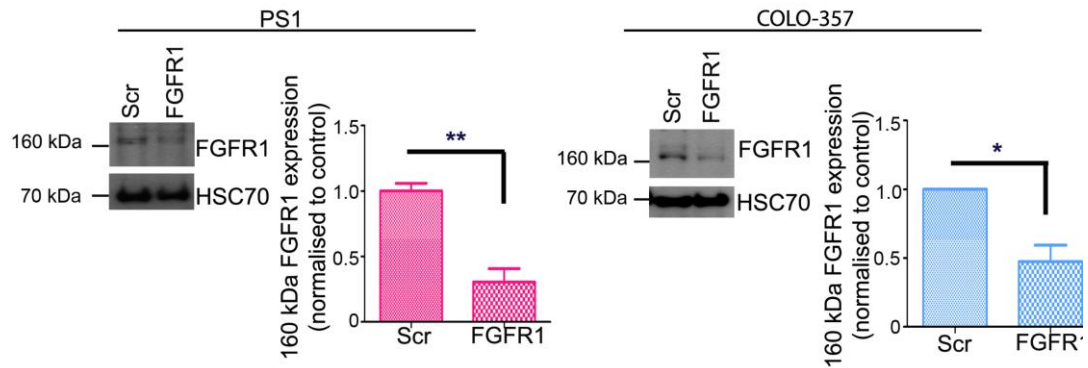


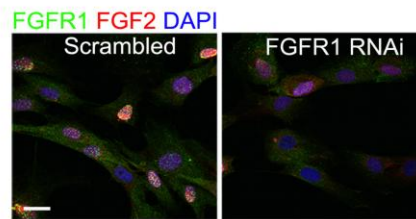
Figure 4. 10 Effect of FGF2 RNAi in PDAC cells

(A-D) MIA PaCa-2 and COLO-357 cells were treated with FGF2 siRNA for 72 hours and stained with antibodies to FGF2 and FGFR1. Treatment with FGF2 siRNA resulted in significant reduction in FGF2 expression but no effect was seen on nuclear FGFR1 levels. Scale Bar: 20 μ m. (E) COLO-357 cells were treated with FGF2 RNAi for 72 hours and subjected to sub-cellular fractionation. No effect on FGF2 knock-down was seen on nuclear FGFR1 expression. * $P < 0.05$, ** $P < 0.01$, Student's t-test. Data summary represented by mean \pm SEM. Scale Bar: 20 μ m. Images are representative of at least three independent experiments.

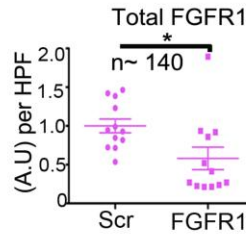
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B



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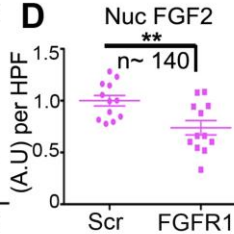


Figure 4. 11 FGFR1 knock-down results in a failure of FGF2 to translocate to the nucleus in stellate cells.

(A) PS1 and COLO-357 cells were transfected with FGFR1 siRNA or scrambled non-targeting siRNA (Scr) for 72 hours and subsequently immuno-blotted to analyse expression levels of FGFR1. The ratio of full length FGFR1 (160 kDa) to HSC70 was determined by densitometric analysis. (B-D) FGFR1 RNAi resulted in significant reduction in nuclear FGF2 compared to scrambled RNAi (Scr) treated PS1 cells. **P<0.01 * P<0.05, Student's t-test. Data summary represented by mean ± SEM. For. For analysis of nuclear FGFR and FGF2 each data point shown represents an average of total or nuclear FGFR or FGF2 per field. Several fields were counted per experiment. The total number of PS1 cells analysed is recorded in the figure (n). For all data, images are representative of three independent experiments.

4.2.10 Effects of blocking FGFR signalling on nuclear FGFR1 and FGF2 in pancreatic stellate cells.

In order to test whether nuclear FGFR1 and FGF2 were dependent upon FGFR signalling, I treated cells with a well validated chemical inhibitor of FGFR1, PD 173074. PD 173074 is an ATP competitive inhibitor of the FGFR family, and shows 1000 fold more selectivity for FGFR1 than other tyrosine kinases such as PDGFR²⁰². PD 173074 has been used successfully *in vitro* and *in vivo* to block FGFR signalling. Pardo and colleagues showed that PD 173074 can inhibit small cell lung cancer growth by blocking FGF2 specific FGFR activation³¹³

Preliminary results, to test the effect of different concentrations of PD 173074 inhibitor (2nM to 2µM) on stellate cells, showed a dose dependent effect on inhibiting nuclear FGFR1, with the most significant effect at 2µM (Figure 4. 12). Treatment of stellate cells with higher doses of the inhibitor (20µM) resulted in substantial toxicity, with only a few viable cells remaining attached to the culture plate. Given that treatment with 2µM PD 173074 resulted in the most significant effect on nuclear FGFR1 and inhibition of FGFR signalling, and that at this concentration PD 173074 is ineffective at inhibiting other RTKs (such as PDGF and EGFR²⁰²), I chose this concentration for subsequent experiments. Confocal microscopy and sub-cellular fractionation showed that treatment with PD 173074 resulted in a significant reduction in nuclear FGF2 (both LMW and HMW) and FGFR1 in PS1 cells (Figure 4. 12). However, no effect on nuclear FGFR1 was seen in COLO-357 cells (Figure 4. 12).

To ensure that the PS1 cell line exhibited a standard response to FGF2 stimulation, serum-starved stellate cells were stimulated with 100 ng/ml FGF2 in the presence of

300 ng/ml heparin in serum-free media for 15, 60, and 120 min. Western blotting, using antibodies specific to phosphorylated FRS2 and extracellular signal-regulated kinase (ERK), confirmed that stimulation with FGF2 activated the FRS2 ERK pathway in PS1 cells (Figure 4. 13). FGF2 treatment triggered rapid FRS2-ERK phosphorylation, and this response was blocked by pre-treatment with PD 173074 (2 μ M) for 1 hour. Of note, abolishing FRS2 using RNAi showed a significant effect on both nuclear FGFR1 and FGF2, confirming that receptor activation is important for nuclear translocation of both receptor and ligand (Figure 4. 13).

To assess whether exogenous FGF2 was able to induce nuclear FGFR1 and FGF2, I treated serum starved PS1 cells with recombinant FGF2 (100ng/ml) in the presence of 300 ng/ml heparin in serum-free media, over 2 hours. Immunostaining revealed that exogenous FGF2 stimulation significantly induced nuclear accumulation of both FGFR1 and FGF2 in stellate cells, within 15 minutes of treatment (Figure 4. 14).

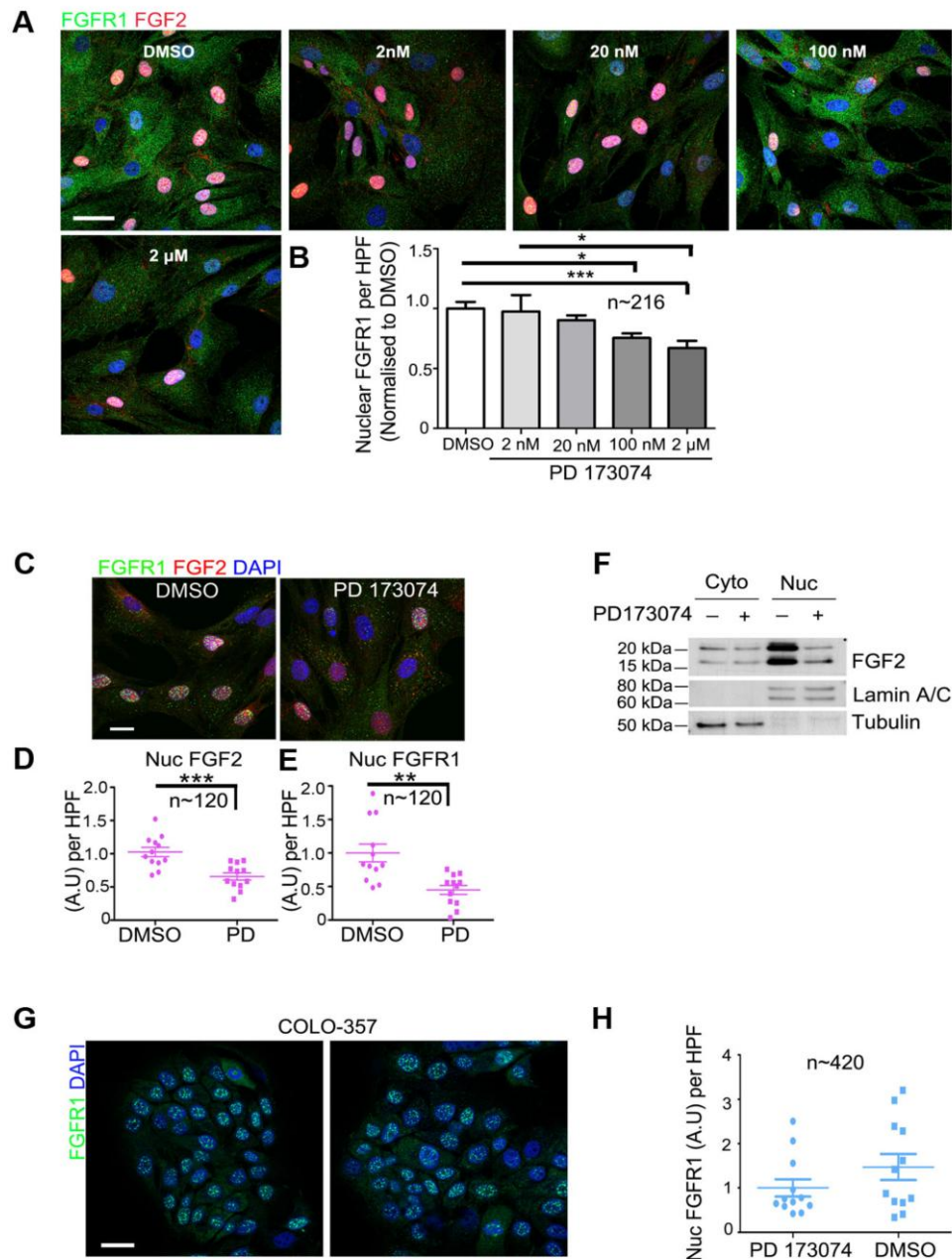


Figure 4. 12 Effect of blocking FGFR signalling on nuclear FGFR1 and FGF2 in stellate cells

(A) Treatment with increasing concentration of PD 173074 (2nm to 2µm) leads to a significant reduction in nuclear FGFR1. (B) Quantification following increasing concentrations of PD 173074 shows significant reduction of nuclear FGFR1 after 100nM and 2µM PD 173074 treatment, compared to DMSO control. (C-F) FGFR inhibitor treatment (PD 173074, 2 µM, 48 hours) resulted in significant reduction in nuclear FGF2 and FGFR1 as compared to vehicle control (DMSO). (G-H) COLO-357 cells were treated with PD 173074 for 48 hours and stained with antibodies to FGFR1. No effect was seen on nuclear FGFR1. Scale Bar: 20 µm. *P< 0.05, **P <0.01, ***P<0.001. Student's t-test. Data summary represented by mean ± SEM. Images are representative of at least three independent experiments.

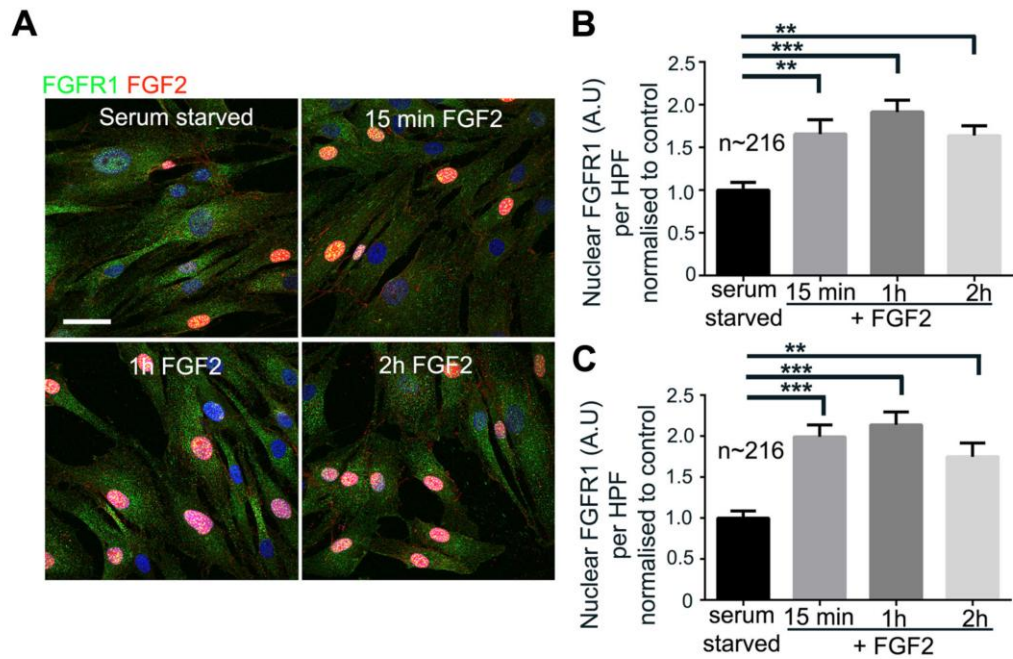


Figure 4. 14 FGFR stimulation induces nuclear FGFR1 and FGF2.

(A) PS1 cells were serum starved for 12 hours and subsequently stimulated with exogenous recombinant FGF2 (100µg/ml) for 2 hours. (B-C) After 15 minutes of stimulation, there was a significant increase in both nuclear FGFR1 and FGF2 compared to serum starved cells. Scale Bar: 20 µm. ***P<0.001, **P<0.01, Student's t-test. Data summary represented by mean ± SEM. Images are representative of at least three independent experiments

4.2.11 FGFR1 localises at nuclear speckles in pancreatic stellate cells.

Immunostaining for FGFR1 in stellate cells demonstrates a distinct speckled pattern within the nucleus. The exact role of nuclear speckles is still controversial, however strong evidence suggest that these sights are centres for RNA Pol II-mediated transcription as well as co-transcriptional, pre-mRNA processing³¹⁴. Indeed, in rapidly proliferating medullary cells, FGFR1 has been shown to localise to these domains, where FGFR1 associated with sites of active transcription and hyperphosphorylated RNA Polymerase II³⁰⁰. Thus, I co-stained stellate cells with antibodies to FGFR1 and the spliceosome assembly factor SC-35, a validated marker of nuclear speckles³¹⁵, to investigate if FGFR1 localised to these nuclear domains in stellate cells.

As previously described, SC35 antibodies showed characteristic staining of speckled sites throughout the nucleus. The speckled sites of staining in the nucleus were also highly enriched for FGFR1, resulting in a yellow colour in the co-localised regions (Figure 4. 15). This is best demonstrated by an overlap of peaks within the nucleus of the confocal profile plot (Figure 4. 15).

4.2.12 Nuclear FGFR1 and FGF2 regulate proliferation of pancreatic stellate cells.

Given that nuclear FGFR1 is a characteristic of rapidly proliferating glioma cells³¹⁶ and that I confirmed FGFR1 localised at nuclear speckles in stellate cells, this prompted me to investigate the effect of nuclear FGFR1 and FGF2 on proliferation. RNAi knock-down of FGF2 or FGFR1 for 72 hours in stellate cells resulted in a significant reduction in the number of cells staining positive for the proliferative

marker Ki67, as well as a significant reduction in total cell count (Figure 4. 16). Furthermore, in agreement with their role in driving proliferation, there was a significant correlation between nuclear FGF2 or FGFR1 and stellate cells that were positive for FGFR1 Ki67.(Figure 4. 17). Of note, no effect was seen on the proliferative index (% Ki67 positive cells or cell count) in cancer cells following FGF2 or FGFR1 knock-down (Figure 4. 18). This suggests nuclear FGFR1 is not required for the proliferative capacity of cancer cells, but together FGFR1 and FGF2 may drive cell growth specifically in stellate cells.

Nuclear FGFR1 has been shown to activate expression of a number of genes, mediated by extracellular signals and their secondary messengers^{304, 306, 307}. *FGF2* was one of the first genes identified as regulated by FGFR1³⁰⁷. It is possible that nuclear FGFR1 may drive proliferation by modulating *FGF2* gene expression. Thus, I performed FGFR1 knock-down on stellate cells and cancer cells to assess the effect on endogenous FGF2 protein levels. Abolition of FGFR1 resulted in a significant reduction of FGF2 (HMW) in PSCs, but no effect was observed in cancer cells (Figure 4. 16 and Figure 4. 18).

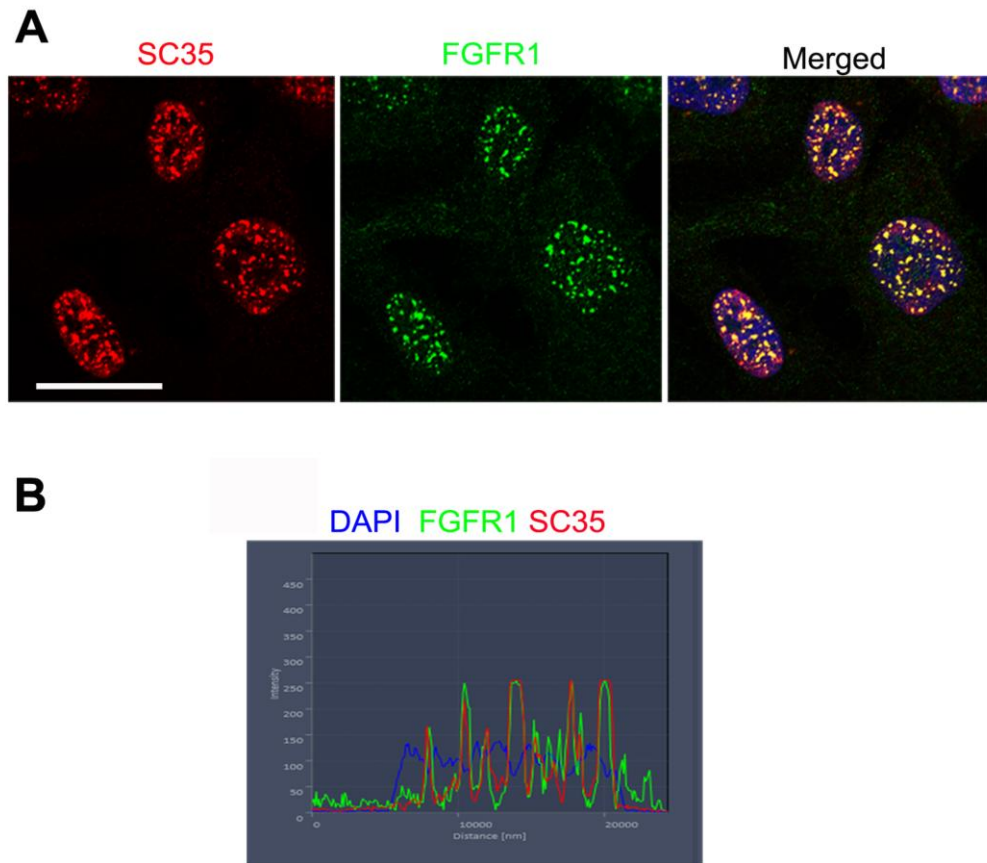


Figure 4. 15 FGFR1 localises at nuclear speckles

(A) FGFR1 (green) co-localised with splicing assembly factor, SC35 (red) at distinct nuclear speckles within the nuclei of stellate cells. Pixel intensity analysis confirmed this co-localisation, with perfect overlap of red and green staining signals within the nucleus (blue). (B) This co-localisation was best visualised by the spatial coincidence of discrete peaks of green and red fluorescence intensity, given by a confocal profile. By taking a 20 μ m transect across the nucleus (blue line), red and green peaks overlapped, with just a few small green peaks that were outside of the nuclear speckles. Co-localisation was determined based on at least three independent experiments

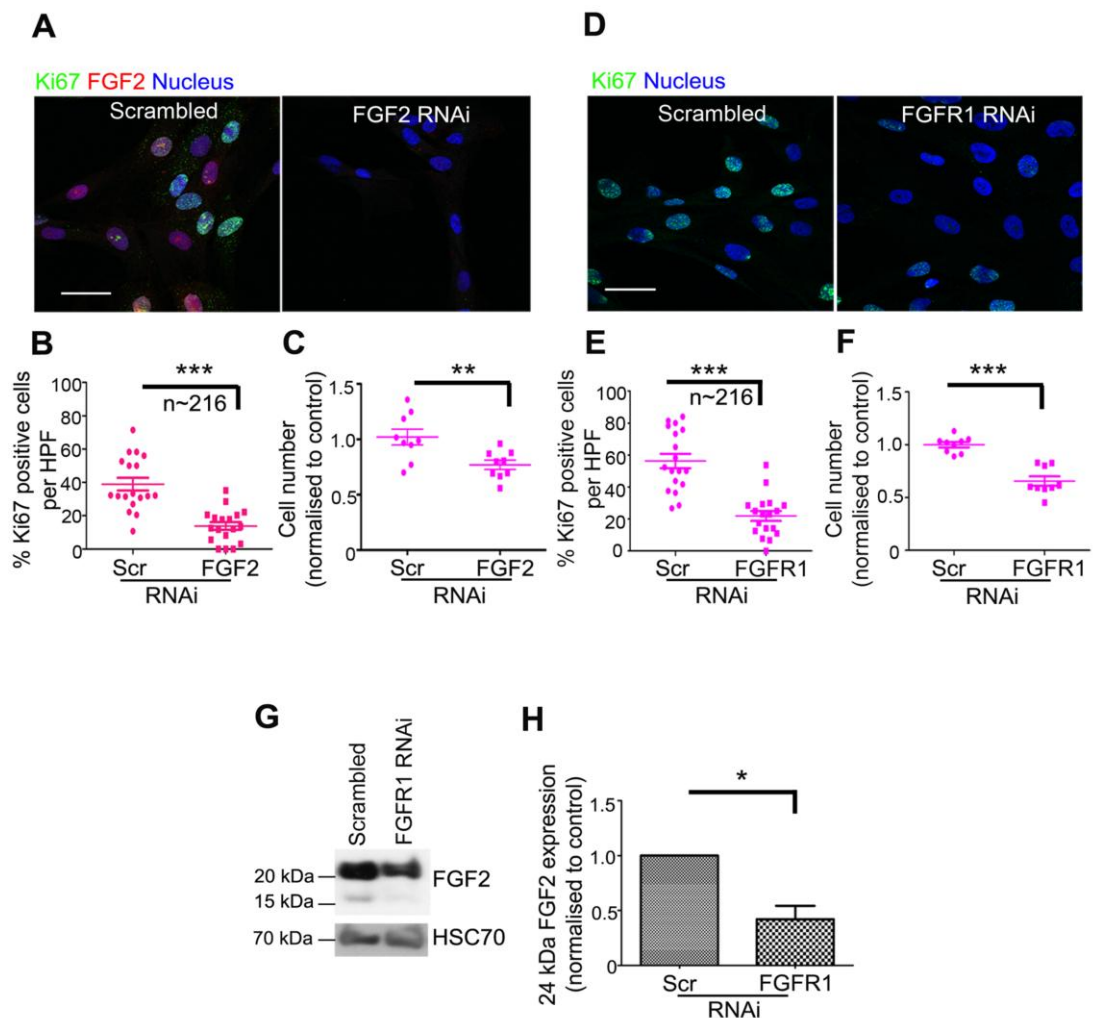


Figure 4. 16 Stellate cell nuclear FGFR1 is associated with PSC proliferation.

RNAi-mediated knock-down of FGF2 (A-C) and FGFR1 (D-F) in stellate cells resulted in a significant reduction in proliferative index (% Ki67 positive cells, B, E, each data point represents percentage of cells positive for Ki67 per field, multiple fields were taken per experiment. A total of ~216 PS1 cells were analysed.) and, consequently, total cell count (C, F, each data point refers to one technical repeat. Three technical repeats were carried out per experiment, each experiment was carried out in triplicate), relative to scrambled RNAi control (Scr). (G-H) RNAi-mediated knock-down of FGFR1 in stellate cells resulted in a significant reduction in FGF2 expression (HMW form). Scale Bar: 20 μ m. ***P < 0.001, **P < 0.01, *P < 0.05, Student's t-test. Data summary is represented by mean \pm SEM. For all data, images are representative of three independent experiments.

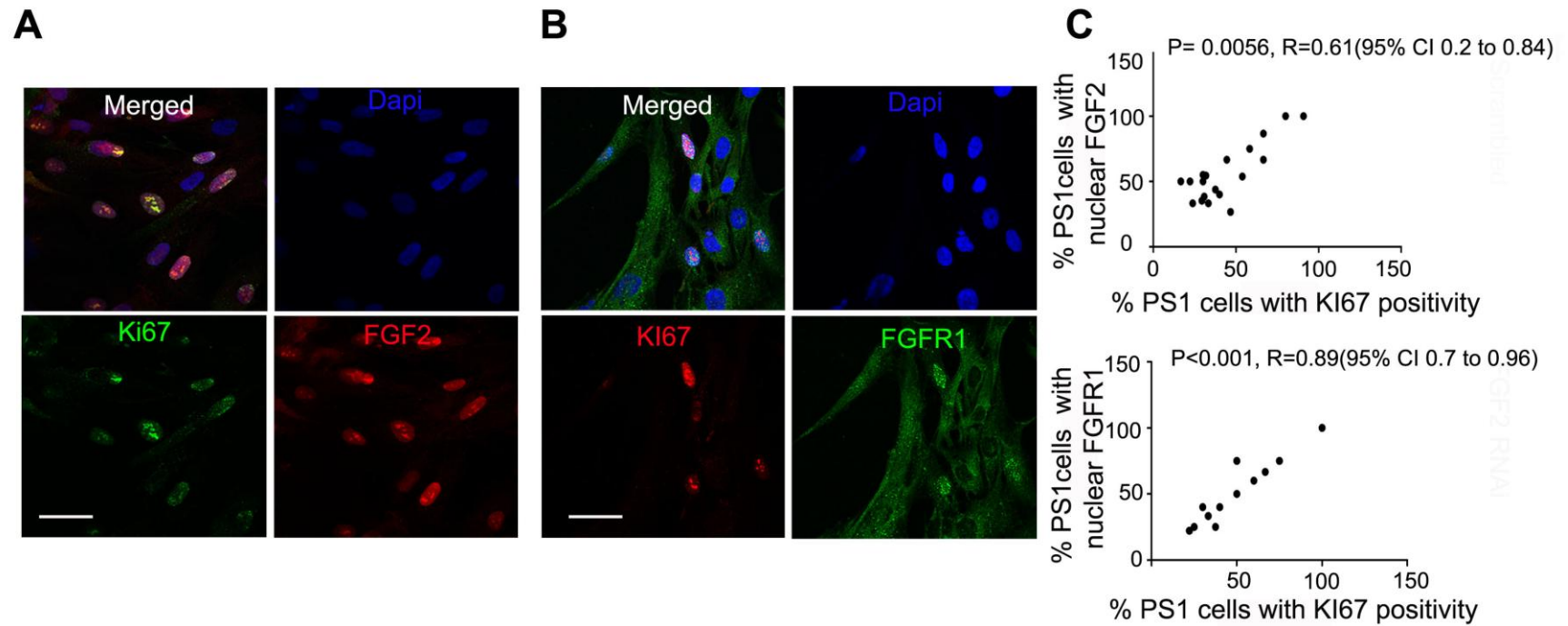


Figure 4. 17 Nuclear FGFR1 and FGF2 correlate with Ki67 positivity in PSCs

(A) Stellate cells that were Ki67 positive (green) also showed nuclear FGF2 (red). (B) Similarly, all cells that were Ki67 positive (green) showed nuclear FGFR1. (C) Graphs show significant correlation between percentage of PS1 cells with nuclear FGF2 and Ki67 as well as percentage of PS1 cells with nuclear FGFR1 and Ki67. Scale Bar: 20 μ m. Data summary represented by mean \pm SEM. Images are representative of at least three independent experiments

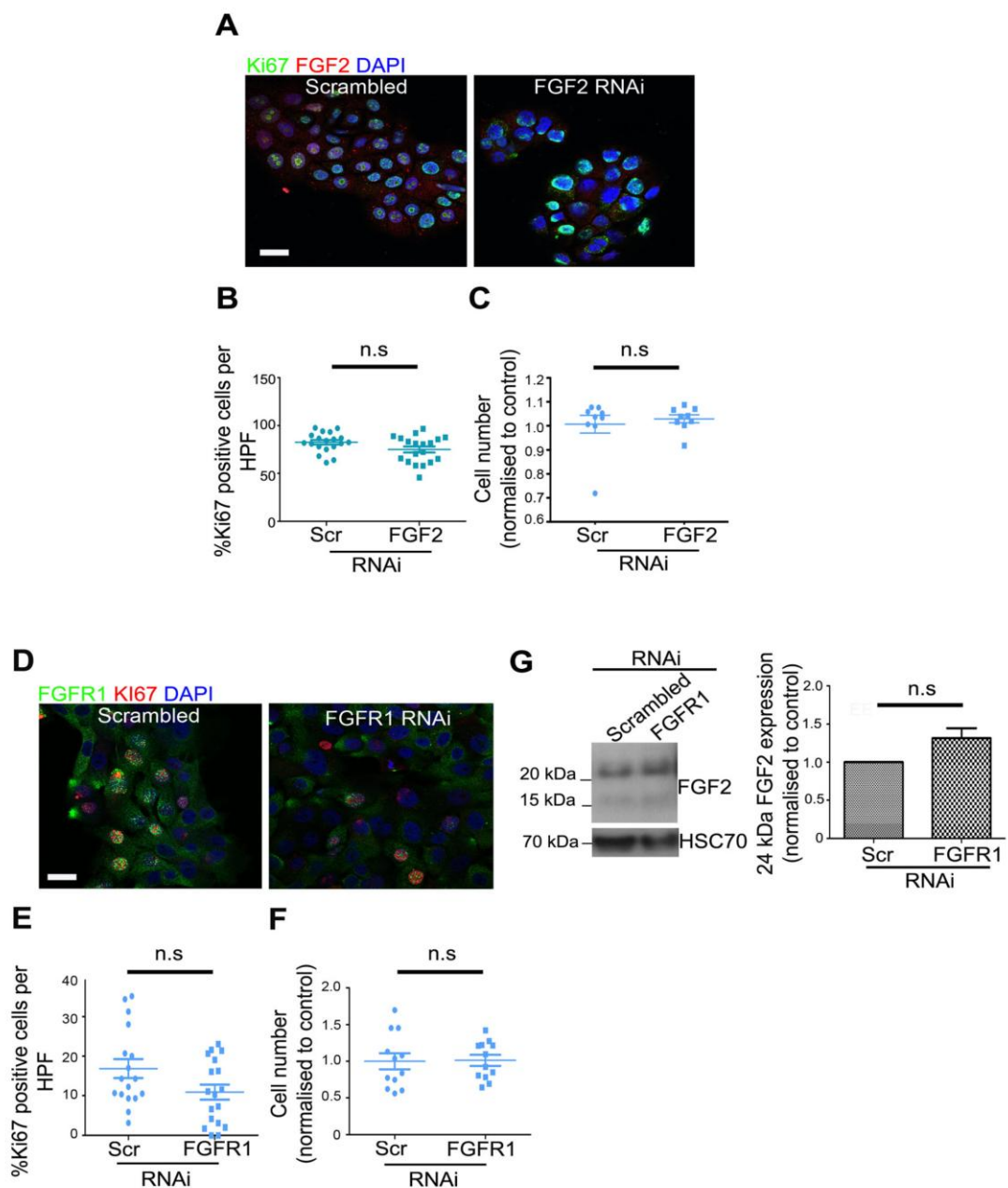


Figure 4. 18 Cancer cell proliferation following FGF2 and FGFR1 knock-down.

(A-C) FGF2 RNAi in COLO-357 cells had no effect on proliferative fraction (percentage Ki67 positive cells: green) or total cell number. (D-F) FGFR1 RNAi in COLO-357 cells had no effect on proliferative fraction (percentage Ki67 positive cells: green) or total cell number. (G) FGFR1 knock-down had no effect on expression of either HMW (24 kDa) or LMW (18 kDa) isoforms of FGF2 compared to Scr RNAi at 72 hours post-transfection in COLO-357 cells. Scale Bar: 20 μ m. n.s. not significant. Data summary represented by mean \pm SEM. Images are representative of at least three independent experiments.

4.2.13 Effect of PD 173074 treatment on cell cycle in pancreatic stellate cells

Next, I wanted to assess the effect of PD 173074 treatment on stellate cell proliferation, since this inhibitor showed a significant effect on nuclear FGFR1 and FGF2 translocation. Treatment of stellate cells over five days with PD 173074, resulted in a reduced increase in cell number compared to treatment with vehicle control (DMSO) within 48 hours and was significantly inhibited by 72 hours of treatment (Figure 4. 19). This reduction in cell number increase correlated with a significant reduction in stellate proliferative index (percentage of Ki67 positive cells) after 48 hours of PD 173074 treatment. These changes in proliferation following treatment with PD 173074 were associated with a reduction in the major G1 cyclin, cyclin D1, only in stellate cells (accompanied by a significant reduction in percentage of cells in G2/M phase and accumulation in the G1 phase of the cell cycle (Figure 4. 20). Once more, PD 173074 treatment had no effect on cancer cells. Rather, treatment with PD 173074 seemed to lead to a small increase in cancer cell growth and proliferation, albeit non-significant (Figure 4. 21).

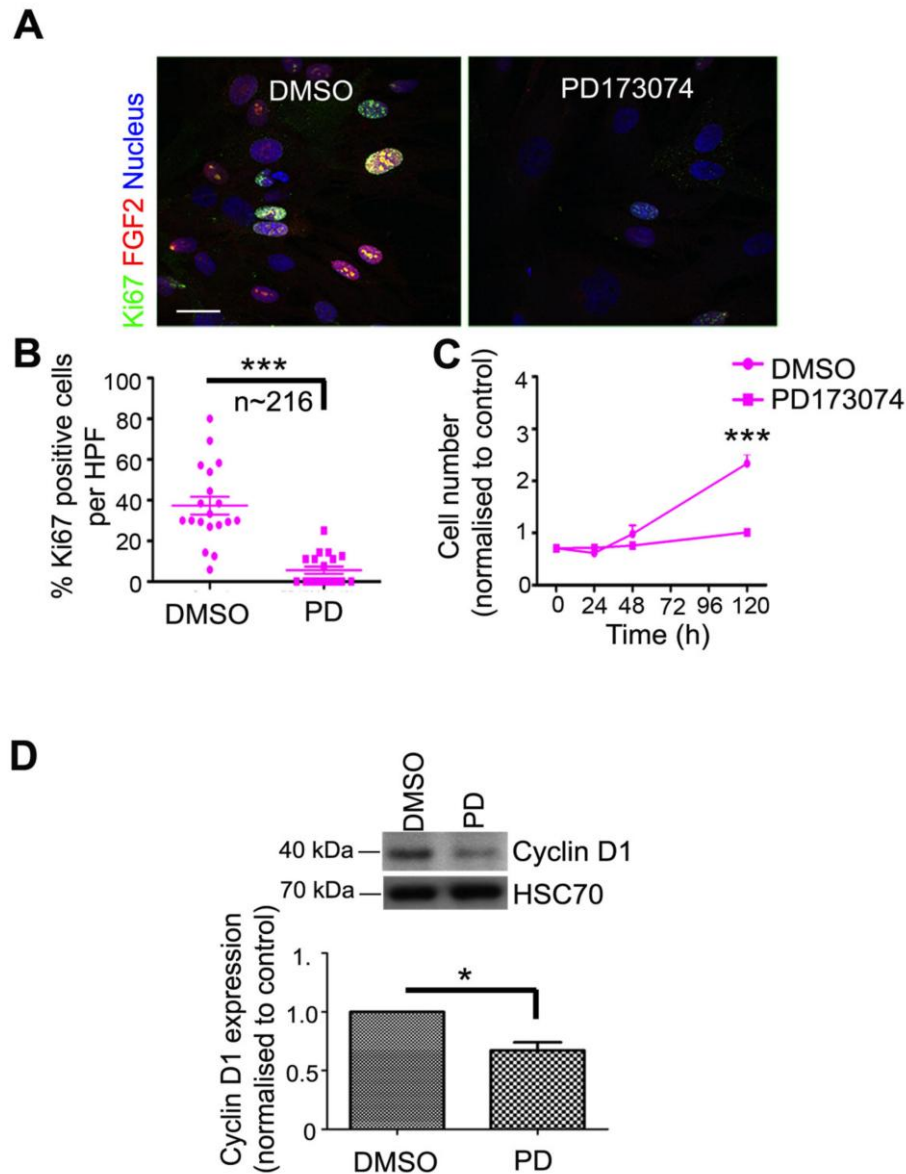


Figure 4. 19 Blocking FGFR signalling results in a reduction in proliferation of PSCs.

(A-C) Drug-mediated inhibition of FGFR (PD 173074, 2 μ M) resulted in a significant reduction in proliferative index (Ki67 positive cells, B, each data point represents percentage of cells positive for Ki67 per field, multiple fields were taken per experiment. A total of ~216 cells stellate cells were analysed.) and cell growth (C, each data point refers to one technical repeat. Three technical repeats were carried out per experiment) after 5 days treatment compared to vehicle (DMSO) treated cells. PD170374 treatment (PD) resulted in significant reduction in Cyclin D1 expression. HSC70 was used as a loading control. Scale Bar: 20 μ m. *** P , 0.001, ** P <0.01, Student's t-test. Data summary is represented by mean \pm SEM. For all data, images are representative of three independent experiments.

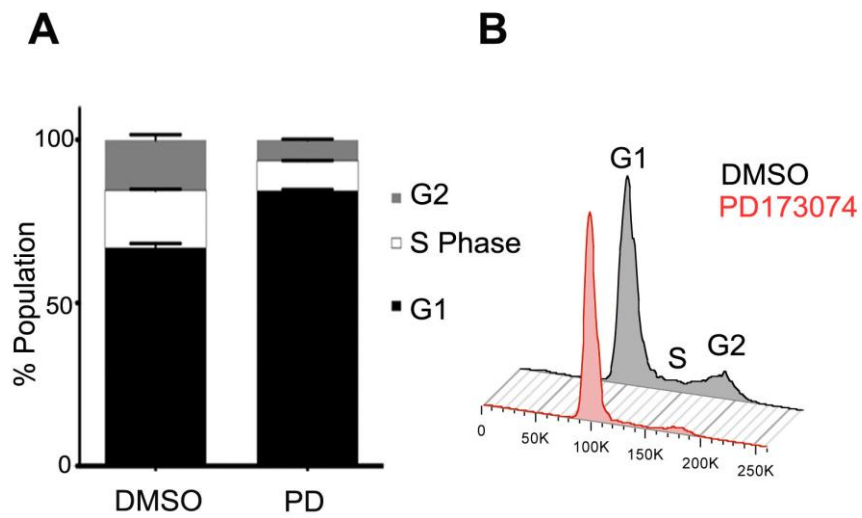


Figure 4. 20 Blocking FGFR signalling results in G1 arrest in PSCs

(A) Cell cycle analysis after treatment with PD170374 (PD) for 48 hours revealed a G1 cell cycle block in stellate cells compared to vehicle-treated (DMSO) cells. Representative cell cycle data after propidium iodide staining and analysis by FACS (B), are shown. Cell cycle data are representative of two independent experiments.

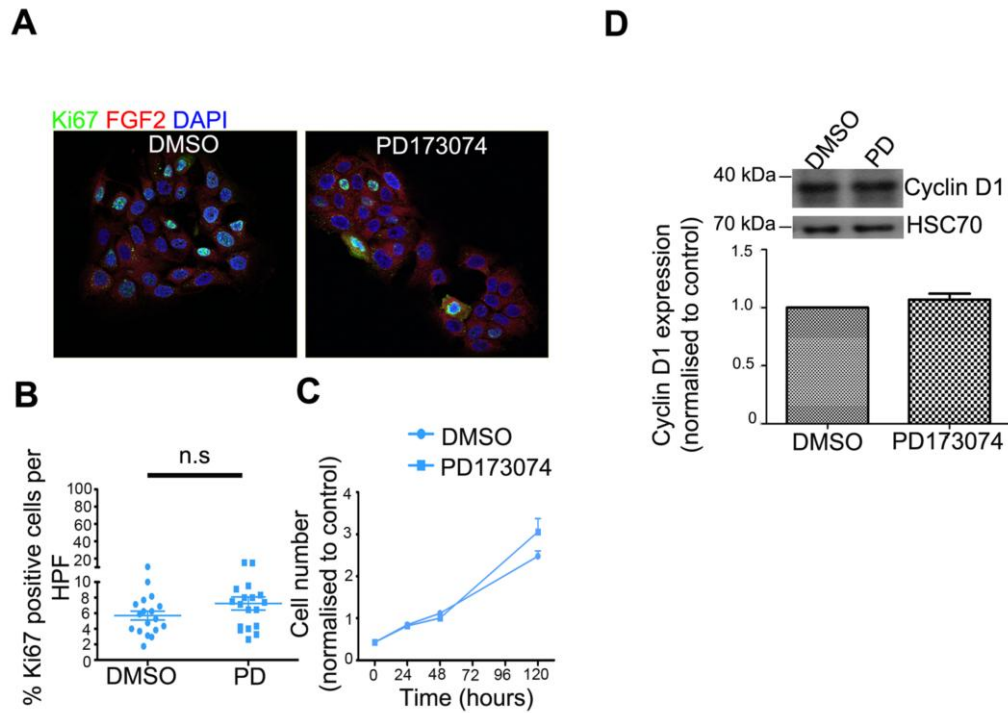


Figure 4. 21 Inhibition of FGFR signalling has no effect on proliferation in cancer cells

(A-C) Chemical inhibition of FGFR1 signalling (PD 173074, 2 μ M) in COLO-357 cells had no effect on proliferative fraction (percentage Ki67 positive cells: green) or cell growth after 5 days treatment compared to vehicle (DMSO) treated cells. (D) FGFR chemical inhibition (PD 173074, 2 μ M, 48 hours), had no effect on Cyclin D1 expression in COLO-357 cells compared to vehicle control. Scale Bar: 20 μ m. n.s. not significant. Data summary represented by mean \pm SEM. Images are representative of at least three independent experiments.

4.3 Discussion

4.3.1 *FGFR1 and FGF2 nuclear localisation in pancreatic stellate cells*

The link between aberrant FGFR signalling and tumourigenesis is clear²⁸². However, much of the work on the functional effect of FGFR signalling in PDAC has come from *in vitro* studies or xenograft models focusing solely on pancreatic cancer cells, despite the stroma representing up to 90% of the tumour volume in this cancer^{88 188, 297}. The pathophysiological mechanisms of tumour stromal progression and therapeutic resistance are still not well understood in PDAC. Previously I have shown that both FGF2 and FGFR1 co-localise to the nucleus exclusively in cancer-associated myofibroblasts *in vivo* (human PDAC), which is associated with tumour invasion. Dissecting the role of intracrine FGFR1 and FGF2 signalling in PDAC has been facilitated by the isolation and creation of PSC cell lines^{124, 169, 199, 317} as well as having access to a panel of PDAC cells. Pancreatic stellate cells are thought to serve as key drivers in the pathobiology of PDAC stroma, where they switch from a quiescent to myo-fibroblastic state^{186, 217, 317}.

Confocal analysis revealed co-localisation of FGFR1 and FGF2 within the nucleus, with staining confined to distinct speckles^{306, 318}. Despite observing nuclear FGFR1 in many cancer cell lines, FGF2 remained predominantly cytoplasmic. Several experiments that I have carried out support the validity of my nuclear FGFR1 and FGF2 data. Firstly, I have used immunostaining combined with confocal microscopy and taking multiple Z-stacking to show that the nuclear interior contained FGFR1 and FGF2. Secondly, using sub-cellular fractionation, I confirmed that full length FGFR1 (~ 160 kDa) and both HMW and LMW FGF2 localised inside the nucleus of stellate cells. This was consistent with other results, which suggest that the full length receptor can localise to the nucleus³⁰⁰. Thirdly, efficient and reproducible

FGFR1 and FGF2 RNAi were able to abolish reciprocal nuclear FGF2 and FGFR1 localisation as shown by confocal microscopy and Western blot analysis. These observations have been supported by other studies that have shown nuclear FGFR1, with an array of different antibodies and with antibodies with different tags, when recombinant FGFR1 was examined^{303, 306, 309, 316}. Finally, translocation of FGFR1 through the nuclear membrane and subsequent nuclear accumulation has also been demonstrated by immuno-electron microscopy³⁰³. Thus the nuclear interior is a major sub-cellular site of functional FGFR1 and FGF2. The observation that FGFR1 and FGF2 colocalise in stellate cells confirms my previous observations in PDAC samples and suggests this relationship may be specific to stellate cell behaviour.

The speckled distribution of FGFR1 within the nucleus appeared strikingly similar to that observed with splicing factors. It is well documented that splicing factors are enriched in regions of the nucleus that have been described as nuclear speckles, based on their staining pattern^{319, 320}. Several non-essential and essential splicing factors are targeted to these domains, including those that belong to the SR repeat containing family of proteins³²¹. In double labeling experiments, I found a very high overlap (>90%) between sites of FGFR1 and the splicing factor SC35 at these speckled domains. In addition to their presumptive role as mRNA processing sites, several studies have established a relationship between speckled domains and sites of transcription³²²⁻³²⁴.

In addition to storage compartments for splicing factors, several studies have documented the association of actively transcribed genes at the periphery and within nuclear speckles. RNA polymerase II and newly synthesized transcripts, labeled with BrUTP, have also been observed at these domains³²⁵. Furthermore, in

rapidly proliferating medulloblastoma human cells, FGFR1 localised to nuclear speckles and associated with sites of pol II transcription but not to sites of DNA replication³⁰⁰. This suggests that sites of transcription, but not replication, associate with nuclear speckles. Although there was near perfect overlap between FGFR1 and nuclear speckles, I did observe a small proportion of FGFR1 located outside the speckled domains. Nuclear speckles have been proposed as storage sites, which could represent sites in which splicing factors and transcription factors are concentrated in large nuclear domains, before being recruited to areas of active transcription³²¹. However, FGFR1 localises together with RNA polIII at speckled domain at similar levels to smaller non-speckled sites suggesting transcription and RNA processing occur concurrently at both nuclear speckles and non-speckled domains³⁰⁰. Thus, it is possible that nuclear speckles in stellate cells may function as at least one of the compartments where transcription may occur via FGFR1 mediated events.

4.3.2 Relationship between nuclear FGFR1 and FGF2

The observation that FGF2 and FGFR1 colocalise in the nucleus of stellate cells, but not cancer cells, suggests there may be a specific relationship between the receptor and ligand, which is not apparent in cancer cells. Indeed, studies have shown that FGFR1 translocates with intracellular FGF2 following cell stimulation³¹⁸. Once in the nucleus, FGFR1 has been shown to activate a number of genes^{306, 307, 318}. Moreover, enhancement of FGFR1 promoter activation is observed after co-transfection of HMW FGF2³⁰⁶. Nuclear accumulation of FGFR1 and FGF2 has been observed in developing brain, neurons, astrocytes, adrenal medullary cells and fibroblasts³⁰⁴. There could be several possible plausible mechanisms for the relationship between nuclear FGFR1 and FGF2 in stellate cells. Firstly, it is possible that nuclear FGFR1 and FGF2 may be internalised as a functional unit and translocate to the nucleus

where they could modulate gene expression. This scenario is possible for FGFR1 and its ligand, as studies have shown that internalised ligand/receptor tyrosine kinase complexes, including EGFR and insulin receptors³²⁶, can retain signalling capacity. Furthermore recent studies by Lin and colleagues showed that crosslinking EGF to EGFR on the cell surface of MDA-MB-468 cells, the ligand receptor complex was found in nuclear extract, and EGFR and EGF formed complexes with chromatin to modulate gene expression²³⁴.

Secondly, it is possible that cytoplasmic FGF2 may control intranuclear FGFR1 mobility and association with active chromatin sites. Studies by Dunham-Ems and colleagues, using FRAP analysis of FGFR1, have shown that there are three possible nuclear FGFR1 populations; a fast mobile population; a slow mobile population, which reflects chromatin bound FGFR1; and an immobile population, representing FGFR1 that is bound to the nuclear matrix and association with actin³²⁷. Two distinct populations of chromatin binding proteins have been described³²⁸. The fast moving population was proposed to represent freely diffusing proteins that undergo rapid non-specific intermolecular collisions, while slower populations represent molecules engaging in binding events and become temporarily immobilised on target genes. FGFR1 can effectively bind to LMW 18 kDa FGF2 (found both extracellularly as well as N-terminally extended predominately nuclear HMW FGF2 (21, 22.5 and 23kDa forms). The same study showed that FGFR1 and HMW FGF2, linked to CFP and YFP, respectively, interact as shown by FRAP³²⁷. Furthermore, HMW FGF2 can decrease the mobility of nuclear FGFR1, following stimulation, to facilitate the interaction of the receptor with gene promoters and other nuclear proteins, such as CBP, thus facilitating transcription³²⁹. Both scenarios may be possible, as I have observed HMW and LMW FGF2 in the nucleus of stellate cells. Whether these isoforms correspond to newly synthesised FGF2 or internalised

ligand is yet to be determined. Either hypothesis requires an interaction between FGFR1 and FGF2 and could explain the reciprocal relationship I have seen in stellate cells. The concept that nuclear FGFR1 and FGF2 may have a functional relationship exclusively in stellate cells was strengthened by the observation that, upon FGF2 abolishment, FGFR1 failed to translocate to the nucleus. However, no effect was observed on nuclear FGFR1 in cancer cells, which do not show nuclear FGF2.

In addition to FGF2 co-localising with FGFR1 in the nucleus, I also observed FGF2 in the nucleolus, a major site of ribosomal synthesis. FGF2 localisation to the nucleolus was first identified in adult bovine aortic cells, suggesting a role in driving quiescent cells into a proliferative state³³⁰. Nuclear localisation of FGF2 in these studies correlated with transcription of ribosomal genes, during transition from G0 to G1 phase of the cell cycle, and increased expression of the major non-histone nucleolar protein, nucleolin, which has a key role in ribosomal transcription²⁵⁸. FGF2 also had a direct effect on the enhancement of RNA polymerase I activity in nuclear extracts isolated from quiescent cells, implying a mitogenic role for nuclear FGF2^{224, 306, 331}. Therefore, it is possible FGF2 (possibly LMW FGF2 that is not targeted for secretion) may be present in different nuclear functional domains to FGFR1, or alternatively may bind to and engage with proteins that may prevent its interaction with the receptor. Thus the nuclear localisation of FGF2 within distinct regions of the nucleus of stellate cells may be driving distinct biological effects in concert with, or without FGFR1.

4.3.3 Abolishing FGFR signalling and its effect on nuclear FGFR1 and FGF2 in pancreatic stellate cells

Upon ligand binding, FGFRs are known to activate several downstream signalling

cascades, with the MAPK pathway the predominant pathway²⁵⁰. To assess whether stellate cells responded accordingly to receptor activation, I stimulated serum-starved cells with FGF2, which activated the FRS2-MAPK signalling pathway rapidly upon stimulation. This pathway was abrogated by pre-treatment with a specific inhibitor for FGFR (PD 173074²⁰²), suggesting that FGF2 specific stimulation, via FGFR, is a strong inducer of MAPK signalling in stellate cells. Having confirmed that FGFR signalling was eliciting the anticipated functional effects in stellate cells, I focused specifically on FGFR1, investigating the sub-cellular trafficking of the receptor after ligand binding. Since the first demonstration that exogenous FGF2 can induce nuclear FGFR1 in fibroblasts³⁰⁵, there have been conflicting reports as to the ability of FGF ligand to elicit nuclear FGFR1 accumulation in various cell types. Other studies have reported that nuclear FGFR1 is stimulated by other factors, such as angiotensin II stimulation, cell depolarization, and protein kinase C, but not exogenous FGF stimulation, in various neuronal cell types³³².

However, using recombinant FGF2 as a known ligand of FGFR1²⁶⁸, I observed dramatic localisation of both FGFR1 and FGF2 to the nucleus, suggesting that FGFR activation is important for nuclear FGFR1 and FGF2 trafficking in stellate cells. Indeed, abolishing FRS2 in stellate cells had a profound effect on nuclear FGFR1 and FGF2. Whether this FGFR signalling occurs at the cell surface, or within the cell, remains to be determined. However, other laboratories have also observed nuclear accumulation of FGFR1 following FGF stimulation, suggesting that receptor activation is required for internalisation and translocation. FGF1, FGF2 and FGF10 stimulation can induce rapid nuclear accumulation of FGFR1 in MCF7 breast cancer cells, and is abolished by pre-treatment with PD 173074 FGFR inhibitor^{309, 333}. Furthermore, nuclear localisation of the EGF receptor in epidermoid carcinoma cells is stimulated by incubation with EGF within 60 seconds of treatment²³⁴. Given

that much of the work that has been carried out on the mechanisms of nuclear FGFR1 is in neuronal cells, it is possible that various cell types respond to stimuli in a cell type specific manner and that ligand stimulation in stellate cells, as with MCF-7 and epidermoid carcinoma cells, which are highly sensitive to stimuli, can elicit nuclear trafficking of its receptor. Furthermore, FGF2 is secreted by stellate cells but not cancer cells. Thus it may fuel an FGFR1 autocrine signalling loop in these cells, stimulating nuclear localisation of FGFR1 and FGF2.

4.3.4 Role of nuclear FGFR1 and FGF2 in driving proliferation of pancreatic stellate cells

Nuclear accumulation of FGFR1 accompanies growth of astrocytes and glioma cells, neurons, differentiation of neuronal progenitor cells and functional changes in adrenal medullary cells, which can be inhibited by transfection of a dominant negative nuclear FGFR1 (TK-) mutant (signal peptide replaced by a NLS)³⁰⁶. Silencing FGF2 or FGFR1, or blocking FGFR signalling with PD 173074, in stellate cells resulted in significant reduction in cell proliferation. These effects were not apparent in PDAC cell lines, which did not display nuclear FGF2, suggesting that nuclear FGFR1 and FGF2 may co-activate genes involved in cellular proliferation exclusively in PSCs. Indeed transfection of mouse fibroblasts with a construct encoding full length FGFR1 with a signal peptide replaced by SV 40 large T antigen NLS, resulted in protein localising exclusively to the nucleus and induced expression of *c-Jun* and *Cyclin D1* following FGF2 stimulation, compared to transfection of full length FGFR1³³⁴. This study suggests FGFR1 stimulation initiates a set of events that are followed by translocation of FGFR1 to the nucleus and regulation of gene expression. C-Jun is a component of the heterodimeric transcription factor AP-1, and is required for cell proliferation³³⁵. C-Jun is also active as a homodimer and its role in proliferation is mediated by direct activation of the *Cyclin D1* promoter³³⁶.

Cyclin D1 is a cell cycle regulator essential for G1 progression, and its overexpression has been shown to shorten G1 and accelerate proliferation²³⁴. Consistent with this, blocking nuclear FGFR1 and FGF2 in PSCs, using PD 173074, correlated with a G1 cell-cycle block and a significant reduction in cyclin D1 expression. Thus activation of cyclin D1 by nuclear FGFR1 and FGF2 may drive entry into the cell cycle, as has been shown in neuronal cells^{224, 226}. Nuclear FGFR1 has been shown to regulate *FGF2* gene expression by activating the *FGF2* promoter³⁰⁷.

Thus, newly synthesized FGF2 in stellate cells could be secreted or could be targeted to the nucleus, fuelling an autocrine signalling loop. Indeed, extracellular FGF2 has been shown to drive astrocyte proliferation and activates quiescent cells from the mature human brain to enter S phase and proliferate²²⁴. Furthermore, in human astrocytes, extracellular FGF2 can stimulate its own expression and drive the synthesis and nuclear accumulation of endogenous FGF2 protein³³⁷. I also observed a significant reduction in FGF2 expression following FGFR1 knock-down, exclusively in PSCs. Thus, modulating FGF2 expression may be another way in which nuclear FGFR1 may regulate proliferation in stellate cells.

Of particular note was the observation that, while the cancer cells I used in my studies express FGFR1, which localises to the nucleus, I did not see any effect of 2 μ M PD 173074 treatment on cell behaviour or nuclear translocation. Furthermore, abolishing FGF2 had no effect on nuclear FGFR1, while RNAi to both FGFR1 and FGF2 had no effect on cancer cell proliferation. Thus it is possible that nuclear FGFR1 is not required to drive proliferation in cancer cells and does not require nuclear cooperation of FGF2. Indeed, pancreatic cancer cells inherently harbour a plethora of genetic mutations, including mutations in *KRAS* and *P53*⁵⁰. An activating

mutation at codon 12 in *KRAS* is detected in close to 100% of all PDACs and in many early neoplastic lesions⁶³. *KRAS* is a small GTPase and potent oncogene that can regulate a number of cellular functions, including survival, proliferation, and differentiation. These diverse activities are driven by *KRAS*-mediated activation of important signalling cascades including the RAF/ERK/MAPK pathway³³⁸. Indeed, both cancer cell lines I used in my studies harboured activating *KRAS* mutations^{192, 195}, which could override the effects of blocking FGFR signalling and/or nuclear FGFR1. This confirms the critical role of nuclear FGFR1-driven proliferation in PSCs, and the importance of analysing stromal cell types in further studies of PDAC behaviour, a characteristic not often taken into consideration in other studies that have tested new drugs solely on cancer cells, demonstrating little or no therapeutic effect.

4.4 Conclusion

Blocking nuclear FGFR1 and FGF2, inhibits proliferation in stellate cells, but not cancer cells. This offers a target selective for stellate cells, which may possibly lead to modulation of cancer cells *in vivo*. The distinct location of FGFR1 at nuclear speckles suggests a role for the receptor as a major regulator of gene expression. Blocking nuclear FGFR1 and FGF2 in stellate cells may influence cancer behaviour by inhibiting transcription of genes that control stellate cell growth and subsequently prevent an environment permissive for cancer cell survival. The results in this section come from experiments performed in 2D culture, which does not reflect the *in vivo* situation. Thus, the next step in my studies was to determine the importance of nuclear FGFR1 and FGF2 in stellate cells in a 3D model of PDAC. Since this model comprises both stellate cells and cancer cells, it allowed me to investigate the effects of paracrine cross talk on cell behaviour in a more relevant environment.

These data suggest a specific role for nuclear FGFR1 and FGF2 in driving PSC proliferation. In contrast to cancer cells, PSCs are exquisitely sensitive to FGFR inhibition, thus opening a new selective therapeutic avenue. The role of tumour-stroma cross-talk is increasingly recognised to play a role in PDAC progression^{81, 169, 170, 203, 217}. Since my observations suggest that targeting the stroma is an attractive option for therapy, next I wanted to use a well-validated, pathologically-relevant PDAC model¹⁷⁰ to specifically assess the interaction of cancer cells and stellate cells. This will be discussed in the next chapter.

4.5 Future challenges.

4.5.1 *Understanding the mechanism of transcriptional control of nuclear FGFR1*

Considering the localisation of FGFR1 and FGF2 in stellate cells at distinct nuclear speckles, it is likely that the receptor can transduce diverse extracellular and intracellular signals directly to the genome. However, whether FGFR1 is a transcriptional regulator in stellate cells is yet to be determined, although these results are the first step into elucidating the molecular mechanism of nuclear FGFR1. Studies have shown that FGFR1 is a potential transcriptional activator, which has been confirmed by chromatin immunoprecipitation experiments^{246, 307}. Based on the study of nuclear FGFR1 in neuronal cells, where it has been shown to regulate growth and differentiation, a proposed 'feed forward and gate' mechanism has been generated for the mechanism of nuclear FGFR1 in these cells³⁰⁴. Diverse stimuli are able to transmit signals to sequence specific transcription factors via classical cytoplasmic nuclear signalling cascades. The nuclear accumulation of FGFR1, which is induced by the same stimuli, feeds the signal forward to CRE binding protein (CBP) to facilitate activation of CBP regulated gene programs³³⁹.

FGF2 was the first gene shown to be regulated by nuclear FGFR1³⁰⁷. *TH*, the rate-limiting enzyme in catecholamine synthesis, has also been shown to be regulated by nuclear FGFR1. *TH* is expressed specifically in catecholamine producing cells. ChIP studies have shown that FGFR1, together with CBP, associates within the promoters of the *TH* and *FGF2* genes³⁰⁶. NF-L is activated, in a cAMP response element (CRE) dependent manner, by nuclear FGFR1³¹⁸. This was shown in differentiating neuronal progenitor cells by transfection of a construct which targeted FGFR1 to the nucleus via an NLS. This increased the expression of both

endogenous NF-L and a CRE containing NFL gene promoter reporter construct. C-Jun and Cyclin D1 have also been shown to be regulated by nuclear FGFR1³³⁴. The promoters of both of these genes contain a CRE element, however it remains to be determined which *cis* elements mediate their regulation by FGFR1. Thus, the regulation of *NF-L* and *TH* genes provides a mechanism through which nuclear FGFR1 may stimulate differentiation of neuronal progenitor cells, while activation of c-Jun and Cyclin D1 may mediate nuclear FGFR1 specific proliferation, as observed in rapidly proliferating glial cells^{224, 226, 318}.

Since previous studies that suggest nuclear FGFR1 may associate with gene promoters, and given the speckled localisation of FGFR1 in stellate cells, I performed chromatin immunoprecipitation sequencing (ChIP-seq) experiments to identify the genomic regions with which FGFR1 may associate. This set of experiments has proved challenging, given the relative lack of knowledge regarding FGFR1 as a transcriptional regulator, and with much of what is known about nuclear FGFR1 being specific to neuronal cell gene regulation. Moreover, results obtained from ChIP-seq experiments, using Illumina ChIP-Seq (Mi-Seq), show that the FGFR1 ChIP-seq data look almost identical to the Input control (negative control), with no enrichment for ChIP reactions that were carried out using anti-FGFR1 antibodies (Appendix). This raises two possibilities, either the antibodies used were not robust enough for ChIP-seq experiments and did not successfully pull down FGFR1 associated chromatin, or the role of the receptor in the nucleus may not be to directly associate with chromatin but may be to function in another manner to regulate cell behaviour (possibly as a scaffold to phosphorylate other protein on the nucleus). However, given the literature and accumulating evidence from other studies on the functional role of RTKs in regulating gene expression, I am optimistic that nuclear FGFR1 plays a similar role in stellate cells. Thus I am currently

screening a panel of FGFR1 antibodies to identify a candidate that may be taken forward as a robust antibody for future ChIP experiments. Furthermore, identifying other nuclear binding proteins in stellate cells that may interact with FGFR1 may also shed light onto the functional role of nuclear FGFR1 and provide a route to understanding how nuclear FGFR1 may elicit its functional role.

If nuclear FGFR1 does not interact directly with chromatin, this begs the question what is the role of nuclear FGFR1 in the nucleus of stellate cells? CREB phosphorylation is critical for CBP, and the closely related p300, to bind to CREB, bridging it with RNA polymerase II complex and modifying promoter associated core histones³⁴⁰. Studies have shown that nuclear FGFR1 can activate CBP and stimulate CREB phosphorylation, which requires a functional receptor transmembrane domain^{306, 307}. CREB can be phosphorylated directly and activated by a number of kinases, including 90 kDa ribosomal S6 kinase (RSK). RSK acts at one terminus of the MAPK signalling cascade and can phosphorylate CREB and histone H3³⁴¹. Activated RSK phosphorylates several transcription factors, including cFos, CREB, I κ B α , the oestrogen receptor and C/EBP. RSK also mediates H3 phosphorylation and thus modulates chromatin remodeling³⁴²⁻³⁴⁸. Using confocal microscopy, FGFR1 was shown to colocalise with RSK1 in nuclear speckles, and FGFR1 can induce RSK1 phosphorylation and increase nuclear RSK1 kinase activity³⁰². Furthermore, transfection of TE671 cells with an FGFR1 expression construct results in FGFR1 accumulating with FGF2 in the nucleus and undergoing phosphorylation³⁰². RSK1 is phosphorylated directly by serine/threonine kinases such as ERK but not tyrosine kinases³⁴⁹. Thus, FGFR1 does not directly phosphorylate RSK1 but may serve as a scaffold that assembles an ERK mediated complex, in which the receptor could activate the RAF/RAS/MEK/ERK cascade and deliver the signal to FGFR1 bound RSK1³⁰². This is a plausible scenario as all

cascade components have been found to be present in the nuclei of stimulated cells³⁵⁰.

Consistent with this model, the authors also observed that transfection of an FGFR1 construct containing a NLS increased ERK phosphorylation in the nuclei of TE671 medulloblastoma cells. Using a GAL4-CREB chimeric construct, the authors also showed that RSK1 stimulates CREB activity and RSK1 activation of CREB is markedly increased by nuclear FGFR1³³². Thus, it is possible that FGFR1 interacts with RSK1, or possibly other nuclear proteins, and mediates their activation, thus impacting on cellular behaviour. This mechanism for indirect activation of gene activity has not yet been interrogated in stellate cells, however it may provide a plausible alternative mechanism for the role of nuclear FGFR1 in driving stellate cell behaviour, given that the full length receptor translocates to the nucleus with FGF2 and is abolished by FGFR signalling inhibition.

4.5.2 The origin of nuclear FGFR1

Furthermore, one of the intriguing questions regarding nuclear FGFR is whether it is derived from the cell surface and how the full-length receptor may transport to the nucleus. There have been several conflicting reports of the origin of nuclear FGFR1. It has been proposed that FGFR1 enters the nucleus by retrograde transport from the ER lumen to the cytosol via the Sec61p channel (a protein complex that mediates co-translational insertion of nascent polypeptides into the ER lumen, as well as providing a mechanism for protein transport in the opposite direction, for example in the ER-associated degradation (ERAD) pathway³⁵¹), before the endoplasmic vesicles deliver the receptor to the plasma membrane³⁵². This process can be viewed as a reversal of integration of membrane proteins into the ER membrane. Proteins structurally similar to FGFR1 (MHC class I molecules, mutant

insulin receptor, or T cell receptor α chain) have been shown to be retrieved from the ER to the cytosol in this manner³⁵³⁻³⁵⁵. The transmembrane domain of the T cell receptor α chain is divided into short polar regions, which are responsible for the release of the receptor from the ER membrane into the cytosol³⁵⁶. Similar to the T cell receptor α chain, FGFR1 consists of non-polar amino acid chains interrupted by polar regions, which may be critical for the mobilisation of the receptor³⁵². The association of FGFR1 TM with the ER membrane is relatively unstable and the nucleus-destined receptor is released into the cytosol before the endoplasmic vesicles deliver the receptor to the plasma membrane. Furthermore, amino acid analysis of FGFR1-4, and predicted conformations, demonstrated that only the TM domain of FGFR4 displayed an uninterrupted α helical structure typical of TM domains. In contrast, the FGFR1 TM domain is likely to consist of a β sheet structure, which is untypical for membrane spanning region. FGFR2 and FGFR3 have TM domains that are intermediate between FGFR1 and FGFR4, with both α helix domains and β sheets³⁵². α helix structures are required by TM domains of most transmembrane proteins for membrane association³⁵². Thus, it is possible that the α helix structure in the TM domain of FGFR1, along with non polar amino acid chains, may be critical for the mobilisation of FGFR1 from the lipid bilayer and subsequent trafficking to the nucleus. This may also provide an explanation why FGFR4 does not traffic to the nucleus³⁵².

In this model, following protein translation, the FGFR1 polypeptide elongates and translocates into the ER lumen. If an atypical transmembrane domain is reached the receptor is released from the ER membrane and FGFR1 is transported into the cytoplasm, otherwise the receptor anchors into the lipid bilayer. Cytosolic FGFR1 may be then degraded by the 26S proteasome, or rapidly transported into the nucleus³⁵². This model was supported by an increase of cytosolic FGFR1 upon

proteasome inhibition. However, studies that propose this mechanism of nuclear entry have not addressed the mechanism for movement of FGFR1 out of the ER membrane into the cytosol, or the impact of stimuli that cause nuclear trafficking of the receptor.

Contrary to this, others have shown that the receptor may originate from the cell surface. This presents a continuing conundrum as to how a membrane receptor can escape the lipid bilayer and traffic to the nucleus. However, studies on EGFR demonstrate that this receptor following ligand stimulation is internalized, targeted for degradation and down regulation of signalling, or other trafficking fates. In some instances, ligand/receptor complexes can initiate signalling from within the endosome³²⁶. Understanding of endocytic trafficking of growth factors is now extending to the nucleus³⁵⁷. Bryant and colleagues, using biotinylation experiments, showed that at least some of the nuclear FGFR1 derives from the cell surface and multiple isoforms of the receptor could be recovered in cell following cell surface biotinylation³³³. Furthermore, the same group has shown that surface FGFR1 is internalised and traffics to the nucleus via the endosomal pathway. Interestingly, inhibition of clathrin dependent endocytosis was not sufficient to block nuclear FGFR1, suggesting another endocytic pathway may be involved³³³. Other studies have suggested a caveolin dependent mechanism for internalisation and nuclear translocation³⁵⁸.

As I have observed that FGFR1 and FGF2 can translocate to the nucleus following ligand stimulation, it is possible to speculate that these proteins will be internalised and jointly translocated to the nucleus. Indeed, studies of EGF family signalling has shown that pairing of EGFR and EGF ligands can act as a functional transcriptional unit within the nucleus itself²³⁴. Once in the cytoplasm, FGFR1 is free to interact with

nuclear import machinery and it may enter the nucleus via importin β ³³⁴. Importin β is soluble nuclear import protein that shuttles between the cytoplasm and nucleus and can carry cargo into the nucleus either directly or with in conjunction with an adaptor protein³⁵⁹.

Recent results from stellate cells suggest that nuclear FGFR1 is mediated by importin β . Co-immunostaining with FGFR1 and importin β showed that importin β is nuclear in stellate cells in which FGFR1 also localises to the nucleus, but remains cytoplasmic when FGFR1 is not nuclear (Figure 4. 22). Furthermore, treatment of stellate cells with importin β RNAi abolishes nuclear FGFR1 (Figure 4. 23). Although these results are preliminary, they suggest that FGFR1 in stellate cells is transported into the nucleus via importin β . Further work is required to understand the origin of nuclear FGFR1 and explain the disparities reported in the literature. Despite the origin of nuclear FGFR1, several questions still remain regarding the role of FGF2 in aiding nuclear entry of FGFR1. Preliminary data from other groups suggest that FGFR1 does not interact directly with importin β ³³⁴, indicating an adaptor molecule is required to mediate the interaction. Most adaptor proteins interact with the NLS of proteins destined for the nucleus. Since FGFR1 lacks a typical NLS³⁰⁴, it is possible that the receptor is co-transported with another molecule containing a NLS. Thus, HMW FGF2, which contains a NLS, may chaperone FGFR1 into the nucleus with importin β . This idea would compliment earlier observations where I observed FGF2 in the nucleus of stellate cells alongside FGFR1 in all experiments.

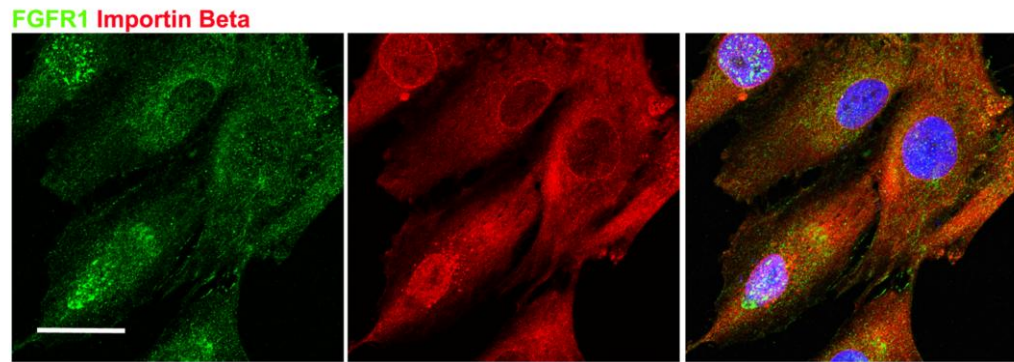


Figure 4. 22 Nuclear FGFR1 and importin β localisation in stellate cells

Immunostaining stellate cells with antibodies to FGFR1 (green) and importin β (red) showed that, in cells in which FGFR1 is nuclear, importin β also localises to the nucleus. In cells in which FGFR1 is mainly cytoplasmic, importin β is not found within the nucleus .

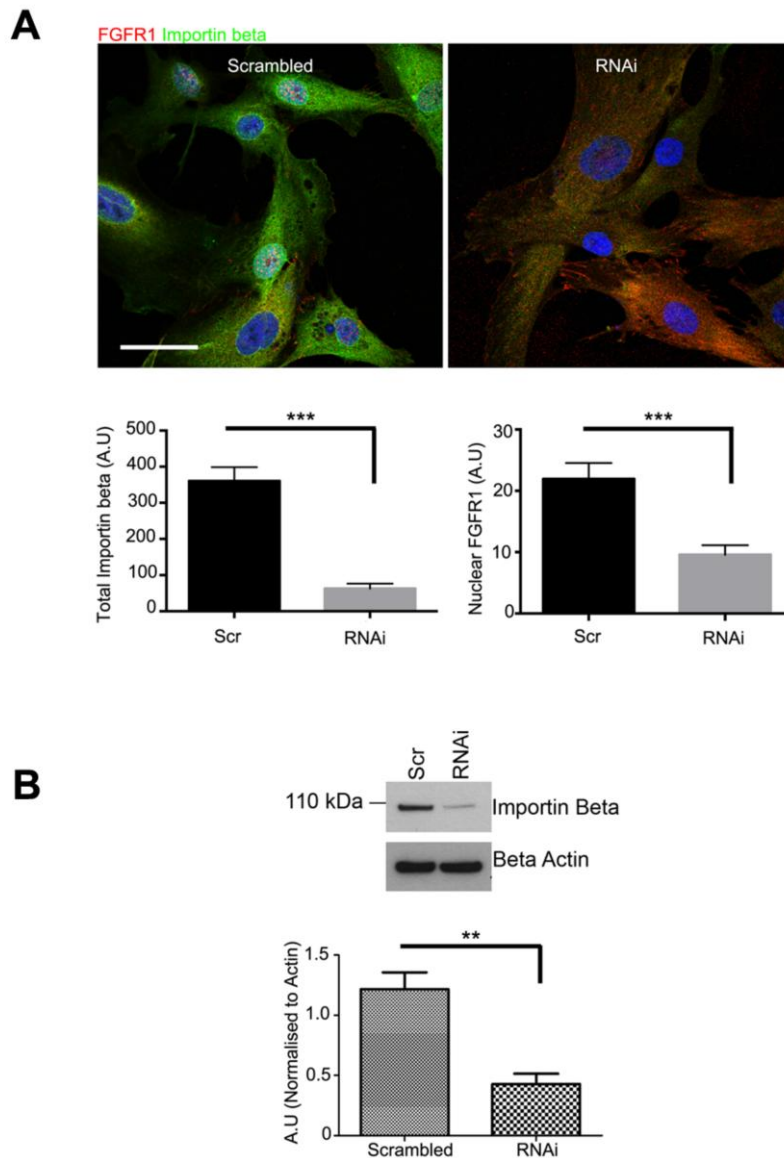


Figure 4. 23 Abolishing importin β , leads to a reduction in nuclear FGFR1

(A) Immunostaining shows that, following treatment of stellate cells with importin β RNAi for 72 hours, there is a significant decrease in importin β expression (green) as well as a failure of FGFR1 (red) to enter the nucleus. (B) Western blotting demonstrates efficient knock-down of importin β after 72 hours treatment with importin β RNAi. Densitometry normalised to Beta actin. Scale Bar: 20 μ m. *** P , 0.001, ** P <0.01, Student's t -test. Data summary is represented by mean \pm SEM. For all data, images are representative of three independent experiments.

CHAPTER V RESULTS-PART III

A physiologically relevant three-dimensional model of PDAC to study the impact of nuclear FGFR1 and FGF2 on pancreatic stellate cell and cancer cell behaviour.

5.1 Introduction.

PDAC is characterised by an intense stroma, which can make up to 90% of the tumour volume. Studies have shown that the tumour stroma cross talk in PDAC can influence chemotherapy response rate and patient prognosis^{360, 361}. Furthermore, pancreatic stellate cells have been found to be the key cell driving the desmoplastic reaction in PDAC¹⁰⁵. PSCs and cancer cells can interact in a bidirectional relationship, activating each other and fueling cell survival, migration and invasion. Therefore, targeting the desmoplastic stromal compartment is of particular interest in PDAC as PSCs are tumour specific, in contrast to circulating immune cells.

I have shown that FGF2 and FGFR1 localise to the nucleus in stromal fibroblasts at the invasive front of human PDAC (Chapter III). Furthermore, results from 2D assays show FGFR1 and FGF2 colocalise to the nucleus in PSCs but not in cancer, or normal ductal epithelial, cell lines. Abolishing FGFR1 and FGF2 functions in stellate cells, using either RNAi or an FGFR inhibitor, results in a significant reduction in cell proliferation and is associated with G1 cell-cycle block (Chapter IV). Therefore, I hypothesised that targeting nuclear FGFR1 and FGF2 in stellate cells may have an anti-tumour effect and offer a novel therapeutic strategy for PDAC.

In cancer research, there has been an abundance of evidence suggesting that 3D models are superior to the conventional 2D culture in plastic flasks. However, current preclinical research still relies heavily on the latter³⁶². From the simplest form: the 'monotypic' cell model, comprising just one epithelial cell type, 3D co-cultures have evolved progressively to contain multiple cell types, thus enabling study of their respective contributions³⁶³. An early example was the 'skin equivalent,' achieved by culturing keratinocytes either on de-epidermalised dermis or on collagen gels embedded with dermal fibroblasts^{364 365}.

The success of pioneering studies with breast epithelial cells cultured in, or on, a reconstituted basement membrane undergoing glandular differentiation, developing apico-basal polarity and forming a central, hollow lumen³⁶⁶, have led to similar experiments for the liver, salivary gland, bone, lung, skin, intestine, kidney and thyroid gland^{364, 367-371}. The choice of cell source and ECM is critical in developing a representative model. For example, human luminal breast epithelial cells grown in laminin-rich basement membrane analogue (Matrigel) form acini³⁷²; however when grown in collagen I, these same cells show an altered integrin profile and abnormal polarity³⁷³.

These 3D models have increased our understanding of how cells perceive biochemical and physical cues from the surrounding microenvironment³⁷⁴. For example, $\beta 1$ integrin is expressed in normal breast epithelial cells but is lost when cells transform into a malignant phenotype. Re-expression of $\beta 1$ integrin in 3D models induces the reversion of the tumour phenotype by allowing the malignant cells to differentiate into glands³⁷⁵.

The incorporation of tissue specific stromal cells is critical for approximation to the *in vivo* condition. Thus, the isolation and availability of human stellate cells has been critical to the development of PDAC organotypic cultures³⁷⁶. The expertise in our laboratory in developing these models has given me the opportunity to understand the role of nuclear translocation of FGFR1 and FGF2 on stellate cell behaviour, and how this may impact on stroma-cancer cell cross talk in a more physiologically relevant system.

5.1.1 Morphological and proliferative response of tumour cells cultured with pancreatic stellate cells on organotypic gels treated with PD 173074.

The introduction of stromal cells in PDAC 3D organotypic cultures was first demonstrated by our laboratory¹⁶⁵. Depending on the hypothesis being explored, the flexible 3D models of PDAC can be set up distinctly. In order to study the invasion of pancreatic cancer cells in the 3D model, I used the 'air-liquid' model, in which cancer cells and stellate cells are cultured on top of an ECM gel (Methods), raised upon a grid and fed from underneath with 2 μ M PD 173074 or vehicle control (DMSO) for 14 days, creating a gradient that stimulates pancreatic cancer cells to invade. Previous studies by our laboratory have shown that the composition and stiffness of the ECM gel can have a profound effect on pancreatic cancer cell behaviour¹⁷¹. When cancer cells and PSCs are co-cultured in ECM gels composed of 75% collagen, 25% Matrigel, cancer cells form multicellular spheroids and recapitulate important features of glandular epithelial cells *in vivo*¹⁶⁵. Thus, all 3D culture experiments were carried out using this matrix composition.

I chose to culture stellate and cancer cells in a 2:1 ratio on top of the gel, as studies by our laboratory have demonstrated that this ratio is optimal for cancer invasion³⁷⁷ and also mimics the *in vivo* situation of PDAC, in which stellate cells often outnumber cancer cells. In order to distinguish between the direct effect of PD 173074 on cancer cells, or an indirect effect via changes in stellate cells, I also plated cancer cells alone on top of the organotypic gels, to compare results with those models in which stellate and cancer cells were admixed.

Haematoxylin and eosin (H & E) staining revealed that when cancer cells were grown alone in organotypic cultures they formed a thin cell layer on top of the ECM gel and failed to invade into the underlying stroma. Furthermore, when cancer cells were plated as a monoculture they showed no significant changes in cell proliferation or invasion upon FGFR blockade (PD 173074, Figure 5. 1). However, when cancer cells were admixed with PS1 cells or primary PSCs, there was a significant increase both in cancer cell number and in invasion of cancer and stellate cells into the matrix. This invasion was abrogated by FGFR blockade (Figure 5. 1), suggesting a pivotal role for PSCs in mediating pancreatic cancer cell invasion. Immunostaining for Ki67, a marker of proliferation, revealed that there was no significant difference in cellular proliferation when organotypic cultures were treated with PD 173074 or DMSO control, neither when they comprised cancer cells cultured alone, or when cancer cells were admixed with PS1 cells (Figure 5. 1)

5.1.2 Invasion of pancreatic cancer cells and stellate cells and the morphological effects of PD 173074 treatment.

When cancer cells and stellate cells were cultured on top of the ECM gel, dual staining of the vehicle treated organotypic cultures, with pan cytokeratin (cancer cells) and vimentin (pancreatic stellate cells), demonstrated that both cancer cells and stellate cells invaded into the ECM gel. Interestingly, stellate cells appeared to invade as either single cells or in collective clusters of cells within one organotypic gel (Figure 5. 2). However, it appeared that cancer cells invaded most commonly as collective cohorts (two or more cancer cells). It also appeared that stellate cell invasion preceded cancer cell invasion and generated tracks for cancer cell invasion to follow, with PSCs exhibiting a spindle fibroblast like morphology (Figure 5. 2). In contrast, in the PD 173074 treated gels, cytokeratin and vimentin staining revealed that stellate cells were 'trapped' within the cancer cell layer on top of the ECM gel and cancer cell invasion was significantly decreased (Figure 5. 2). Interestingly, 'trapped' stellate cells showed a more rounded, 'quiescent like' morphology than the invading stellate cells in vehicle treated gels.

In order to confirm my observations, and not limit them to one cell line, I also used primary pancreatic stellate cells, isolated from cancer-associated tissue, in the 3D culture models. However, the relative paucity of primary stellate cells to conduct all the experiments in sufficient replicates restricted me to using a mini organotypic model (Methods), which has been shown to give comparable results to the conventional 'air liquid' co-culture model¹⁷¹. Using fewer cells per organotypic gel, stellate cells and cancer cells, in a 2:1 ratio, were plated on top of an ECM gel constructed in a Transwell insert, fed from below with medium containing either vehicle control or PD 173074, for 7 days. Confirming earlier observations, H & E

staining revealed that PD 173074 treatment abolished invasion. Again, these observed changes were due to the effects of PD 173074 on stellate cells, as treatment of cancer cells alone in the 3D cultures did not result in any changes in invasion (Figure 5. 3). Dual staining with cytokeratin and vimentin again showed stellate cells failed to invade into the ECM when treated with the FGFR inhibitor. Interestingly, it appeared that, rather than remaining trapped within the cell layer with cancer cells, less invasive primary stellate cells appeared to form a wall at the tumour - gel interface (Figure 5. 3). Thus, the prevention of stellate cell invasion following PD 173074 treatment inhibited cancer cell invasion, possibly due to the decrease in invasion promoting tracts that are formed by stellate cells.

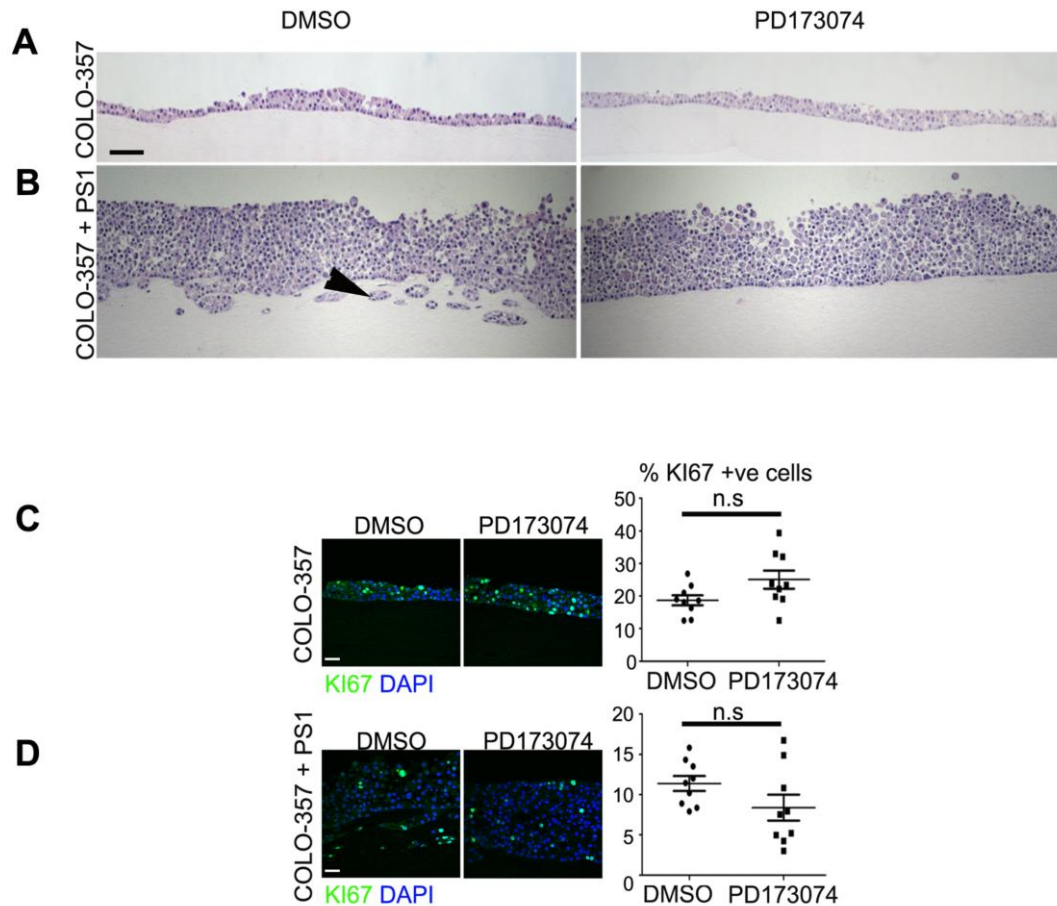


Figure 5. 1 FGFR inhibition in stellate cells leads to reduced cancer cell invasion.

(A-B) A 2x2 experimental design, with COLO-357 cells alone or PS1 and COLO-357 cells co-cultured in the presence or absence of PD 173074 (2 μ M) for 14 days, was used to detect consequences of inhibition of FGFR1 signalling (See Methods). H&E images showed that (A) COLO-357 cells alone formed a thin monolayer on top of the extra-cellular matrix (ECM), and were not affected by FGFR inhibition (PD 173074). (B) In the presence of stellate cells (PS1), there was a marked increase in cancer cell (COLO-357) number as well as invasion (arrow head) into the ECM. This invasion was abrogated by FGFR inhibition (PD 173074). (C-D) There was no significant change in proliferative index (Ki67 staining) in organotypic cultures treated with PD 173074 when PS1 and cancer cells were admixed, relative to when cancer cells were cultured alone. Scale Bar: 100 μ m.*** $P < 0.001$. Student's t-test. Data summary represented by mean \pm SEM or median \pm interquartile range. Images are representative of at least nine organotypic gels for each condition.

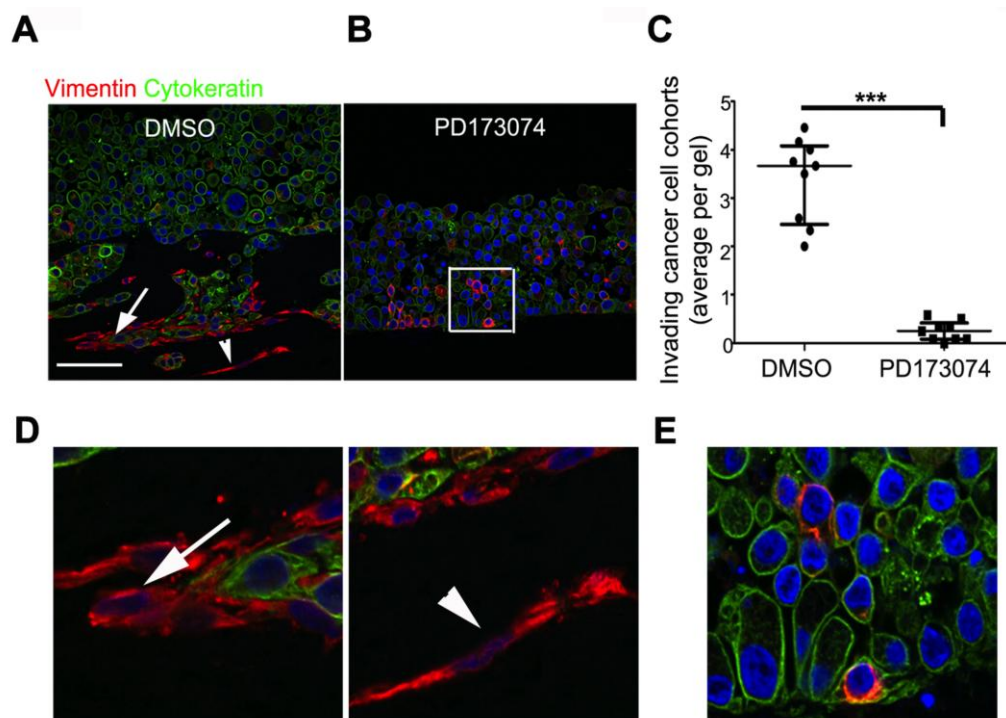


Figure 5. 2 PD 173074 treatment of organotypic culture: changes in stellate cell morphology and invasion.

(A-B) Cytokeratin (green) and vimentin (red) staining to delineate tumour and PS1 cells, respectively, confirmed a significant decrease in cancer cell invasion into the ECM upon FGFR inhibition (PD 173074), compared to vehicle-treated (DMSO) cultures. (C) Graph shows the reduction in cancer cell invasion into the ECM when cultures were treated with PD 173074. Invading cohorts were analysed over twelve fields per organotypic gel. Each data point represents an average of invading cohorts across these 12 fields. (D) Arrow shows stellate cells at the leading edge of invading cancer cohorts, magnified from A. Arrow head shows stellate cells has a more spindle like morphology when invading compared to stellate cells that were trapped in the cell layer. Stellate cells appeared trapped within the overlying cell layer following PD 173074 treatment and failed to migrate into the underlying ECM, magnified in E.. Scale Bar: 100 μ m. *** $P < 0.001$. Student's t-test. Data summary represented by mean \pm SEM or median \pm interquartile range. Images are representative of at least nine organotypic gels for each condition.

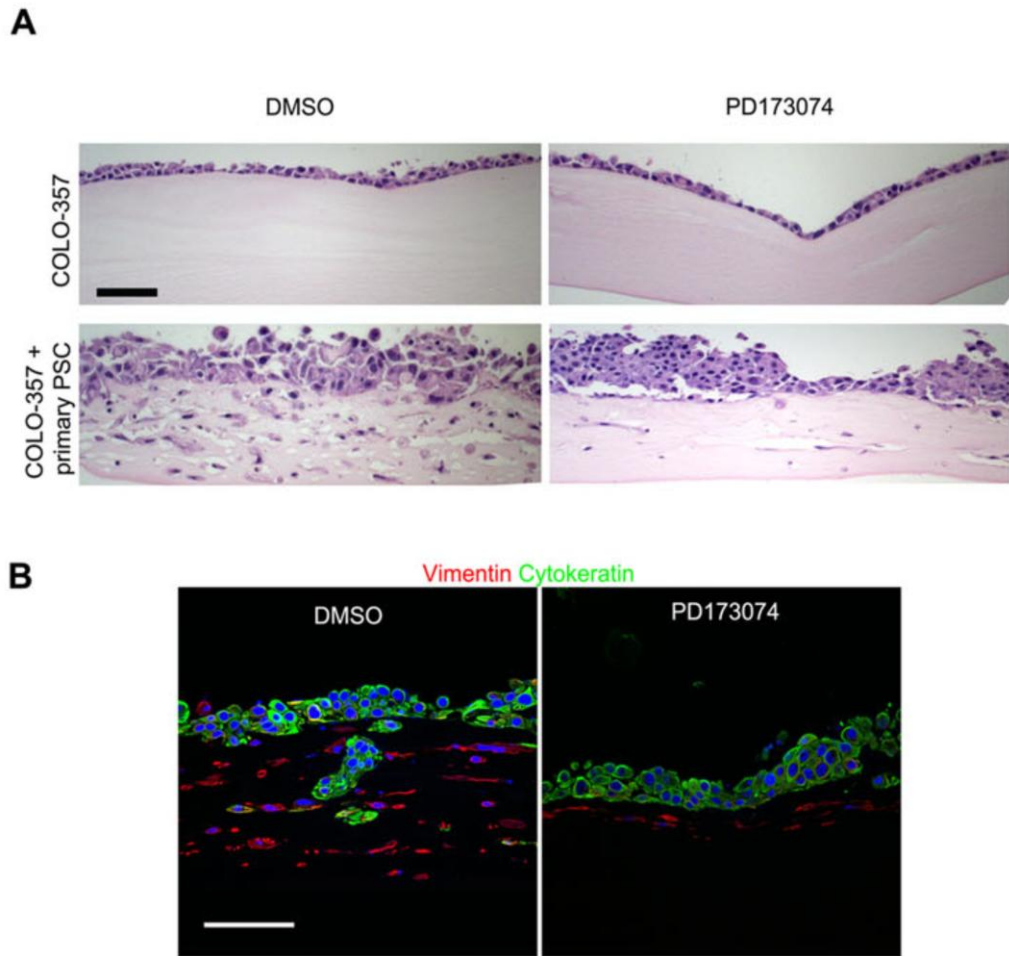


Figure 5. 3 Invasion of primary stellate and cancer cells in a mini-organotypic 3D model.

(A) COLO-357 cells only (or primary PSCs and COLO-357 cells) were cultured in the presence or absence of PD 173074 for 7 days. H&E staining of gel sections showed that COLO-357 cells alone formed a thin monolayer on top of the ECM and were not affected by treatment with PD 173074. When primary PSCs and COLO-357 cells were cultured together there was a marked increase in cell number and invasion into the ECM, which was abrogated by treatment with PD 173074 (B). Vimentin (red) and cytokeratin (green) staining showed that both cancer cells (cytokeratin positive) and primary PSCs (vimentin positive) invaded into the ECM) and this was blocked by PD 173074 treatment. Scale bar 100 μ m.

5.1.3 Blocking stellate cell nuclear FGFR1 and FGF2 abolishes cancer cell invasion in an organotypic model of PDAC.

FGFR1 and FGF2 localise to the nucleus in fibroblasts at the invasive front of PDAC, but not at the centre of the tumour. Furthermore, 2D cell culture based assays have shown that treating stellate cells with 2 μ M PD 173074 leads to a significant reduction in nuclear FGFR1 and FGF2. Thus, I wanted to interrogate the organotypic culture model for localisation of FGFR1 and FGF2 in specific cellular compartments (within both cancer and stromal cells), upon PD 173074 treatment. Immunostaining with FGFR1 and vimentin, to mark stellate cells, showed FGFR1 was localised mainly to the nucleus in invading stellate cells. In contrast, in those stellate cells remaining juxtaposed to cancer cells, it was mainly in the plasma membrane and cytoplasm (Figure 5. 4)

Upon PD173074 treatment. FGFR1 localised to the nucleus in the stellate cells invading into the matrix, whereas those stellate cells remaining juxtaposed to cancer cells showed less frequent nuclear FGFR1 (Figure 5. 4.). Similar results were also demonstrated in organotypic cultures constructed with cancer-associated primary stellate cells obtained from patients (Figure 5. 5). I also confirmed that PS1 cells that were able to invade into the matrix showed significantly more nuclear FGFR1 than trapped cells, using digital quantification to give a more unbiased approach (Figure 5. 5).

A huge advantage of the organotypic system is that any component of the model can be readily modulated in a short time frame. Therapeutic agents such as chemotherapy, small molecules or RNAi can be tested in these organotypic cultures.

PD 173074 is an highly selective FGFR1 inhibitor at nM concentrations, however studies have shown that this molecule can also inhibit phosphorylation and activation of FGFR2, FGFR3 and FGFR4 as well as VEGFR2^{202, 378}. Thus, to confirm specific abolition of FGFR1 could prevent invasion of stellate cells and indirectly affect cancer cell invasion, I admixed PS1 cells, that had been treated with FGFR1 RNAi for 24 hours, and COLO-357 in a 2:1 ratio and cultured them on top of a mini-organotypic gel for 7 days. Compared to cultures generated using stellate cells treated with scrambled control RNAi, knock-down of FGFR1 in stellate cells resulted in a significant reduction in cell invasion (Figure 5. 6). Western blotting over 7 days confirmed that FGFR protein levels were abolished in stellate cells for the duration of the experiment. These results confirm previous observations in organotypic cultures, as well as observations in PDAC tissue, that nuclear FGFR1 and FGF2 can promote stellate cell invasion, and show for the first time that this invasion promoting effect can be inhibited by targeting stellate cells with PD 173074.

In addition, immunostaining for FGF2 and α SMA (to mark stellate cells) revealed there was a significant increase in the percentage of invading stellate cells with nuclear FGF2, compared to non-invading stellate cells. When treated with PD 173074, PS1 cells were unable to invade into the extra-cellular matrix and FGF2 remained cytoplasmic (Figure 5. 7 and Figure 5. 8).

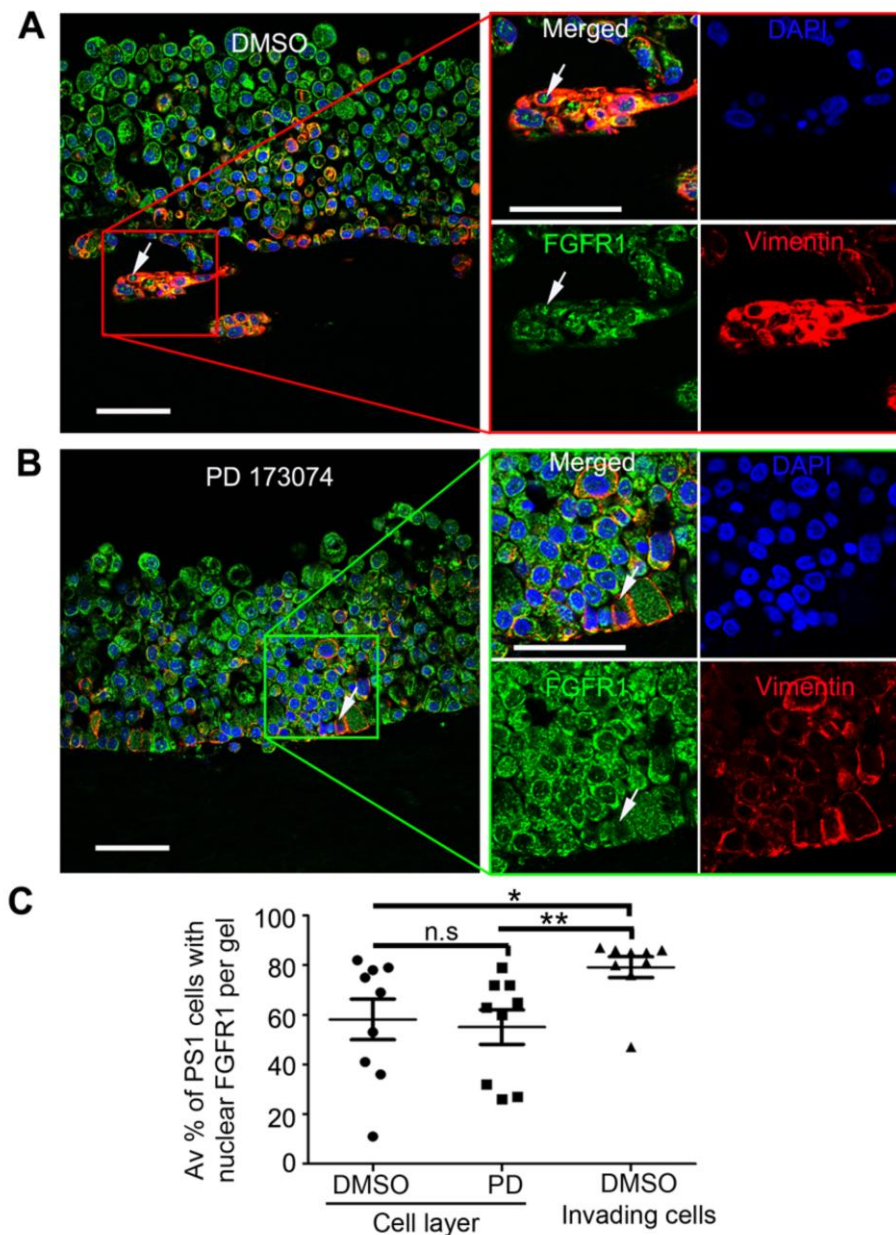


Figure 5. 4 Differential FGFR1 localisation in stellate cells upon FGFR inhibition in 3D cultures.

The percentage of stellate cells (identified by vimentin stain: red) demonstrating nuclear FGFR1 (green) was significantly less in the stellate cells that failed to invade into the extra-cellular matrix) as compared to those invading (arrow in A) in vehicle-treated organotypic cultures. Upon FGFR inhibition (PD 173074), stellate cells failed to localise FGFR1 to the nucleus and did not invade into the matrix (arrow in B, quantified in C). A total of four fields were counted per organotypic gel. Each data point represents an average of the percentage of stellate cells with nuclear FGFR1 over these four fields (total ~540 cells counted in total). Scale Bar: 100 μ m. ** $P < 0.01$, * $P < 0.05$, n.s: not significant. Student's t-test. Data summary represented by mean \pm SEM or median \pm interquartile range. Images are representative of at least nine organotypic gels for each condition.

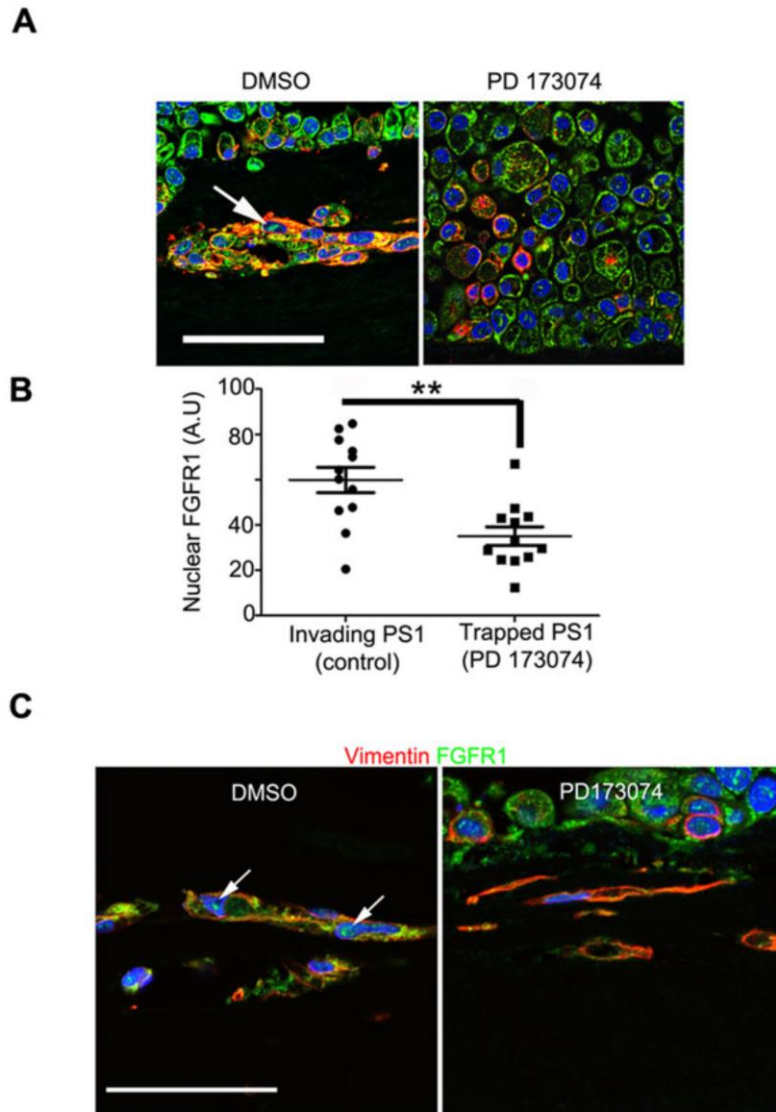
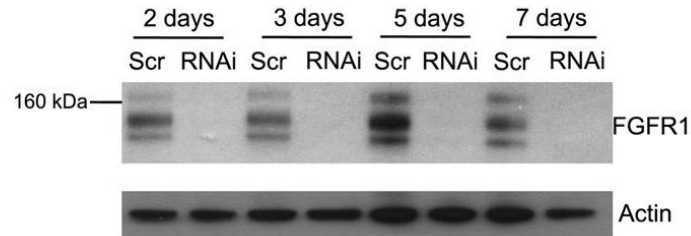


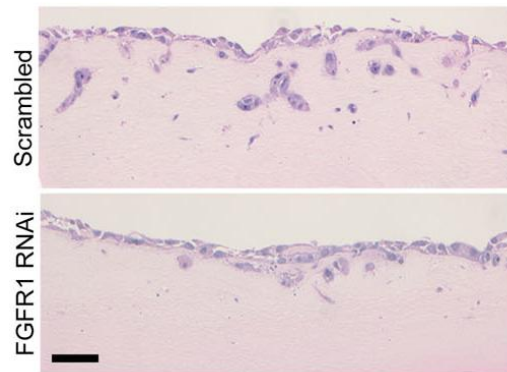
Figure 5. 5 Digital quantification of FGFR1 localisation in stellate cells upon FGFR inhibition in 3D cultures.

Nuclear FGFR was also analysed in vimentin positive invading and ‘trapped’ stellate cells following PD 173074 treatment (A) using Image J digital quantification (B, see methods). Those cells that were able to invade (vehicle treated) showed significantly more nuclear FGFR1 than those cells that remained trapped in the cell layer following PD 173074 treatment. Each data point represents the average nuclear FGFR1 per field. Several fields were counted from three separate gels (a total of ~120 stellate cells per condition were analysed). (C) FGFR1 (green) appears nuclear in invading stellate cells (vimentin positive) when treated with vehicle control compared to stellate cells that failed to invade following treatment with PD 173074. These images are zoomed in to demonstrate the invading stellate cells in the ECM for the DMSO control and stellate cells next to cancer cells in the PD 173074 treated mini-organotypics. Scale Bar: 100 μ m. ** $P < 0.01$. Student’s t-test. Data summary represented by mean \pm SEM or median \pm interquartile range. Images are representative of at least nine organotypic gels for each condition.

A



B



C

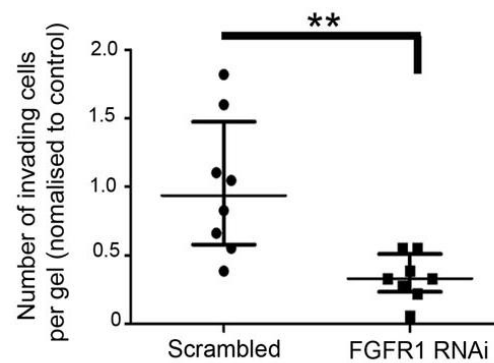


Figure 5. 6 Abolishing FGFR1 in stellate cells prevents cell invasion.

(A) Western blotting confirms that FGFR1 was abolished for 7 days following FGFR1 RNAi treatment compared to scrambled RNAi treated stellate cells. (B) Stellate cells were treated with FGFR1 or scrambled RNAi for 24 hours before harvesting and subsequent culture in a mini-organotypic model admixed with COLO-357 cells in a 2:1 ratio. Gels were cultured for 7 days. H & E images show a significant reduction in total cell invasion when PS1 cells were depleted for FGFR1 compared to scrambled RNAi treated PS1 cells. Number of invading cells is quantified in C., ** P < 0.01, Student's t-test. Data summary represented by mean \pm SEM or median \pm interquartile range. Images are representative of at least nine organotypic gels for each condition.

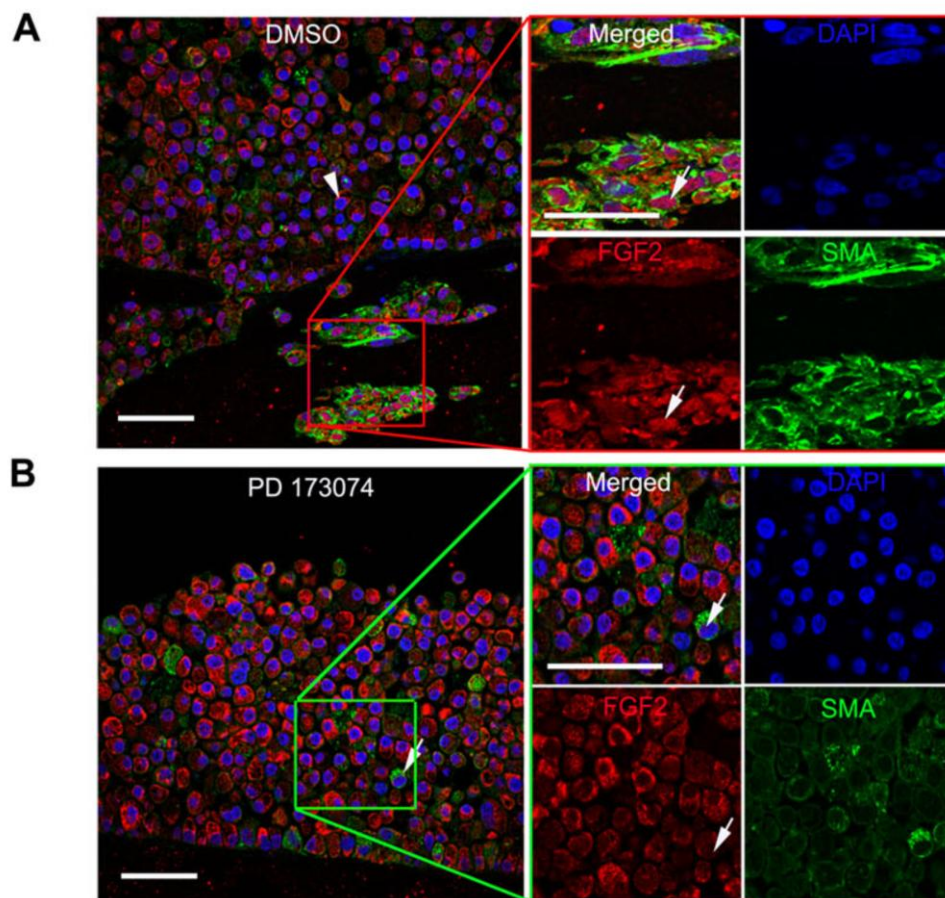


Figure 5. 7 Differential FGF2 localisation in stellate cells upon FGFR inhibition in 3D cultures.

In vehicle-treated organotypic cultures, FGF2 (red) was nuclear in a significant percentage of stellate cells (identified by α SMA stain: green, arrow in A) that invaded into the extra-cellular matrix, compared to those that remained within the admixed cell layer on top of the gel (arrow head in A). Upon FGFR inhibition (PD 173074, 2 μ M, 14 days), stellate cells failed to invade into the ECM. FGF2 was mainly cytoplasmic in these non-invading stellate cells (arrow in B). Scale Bar: 100 μ m.

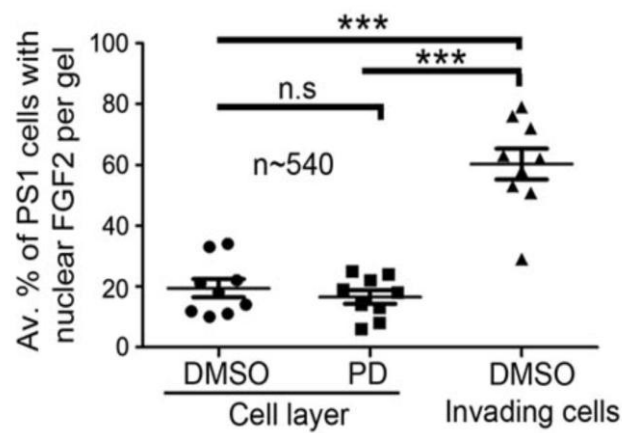


Figure 5. 8 Quantification of FGF2 localisation in stellate cells upon FGFR inhibition in 3D cultures.

Upon FGFR inhibition (PD 173074, 2 μ M, 14 days), stellate cells failed to invade into the ECM. FGF2 was mainly cytoplasmic in these non-invading stellate cells (arrow in B). A total of four fields were counted per organotypic gel and each data point represents an average of percentage of stellate cells with nuclear FGF2 over these four fields (total ~60 cells counted per data point). *** P <0.001. Student's t-test. Data summary represented by mean \pm SEM. Images are representative of at least nine organotypic gels for each condition.

5.1.4 Cancer cells secrete factors that regulate FGF signalling in stellate cells

In a diseased state, under the influence of growth factors, cytokines and oxidative stress, PSCs transform into a myofibroblast like phenotype, secreting excess amounts of ECM, as well as matrix degrading enzymes. Growth factors such as TGF- β 1, PDGF and VEGF, which are secreted by pancreatic cancer cells, are known to induce PSC activation^{121, 122}. These observations support the concept that pancreatic cancer cells recruit stromal cells to produce a growth permissive environment that facilitates cancer progression¹⁵⁵. To determine whether an interaction exists between pancreatic cancer cells and stellate cells, which may influence the FGF pathway, I examined the influence of conditioned medium from the pancreatic cancer cell line COLO-357 on the nuclear translocation of FGFR1 and FGF2 in stellate cells. Incubation of stellate cells with conditioned medium from COLO-357 cancer cells, but not normal pancreatic ductal epithelial cells (Dec-h TERT), led to a significant increase in nuclear translocation of FGFR1 and FGF2 (Figure 5. 9). Thus, nuclear translocation of FGF2 and FGFR1 in stellate cells is regulated by factors secreted by cancer cells.

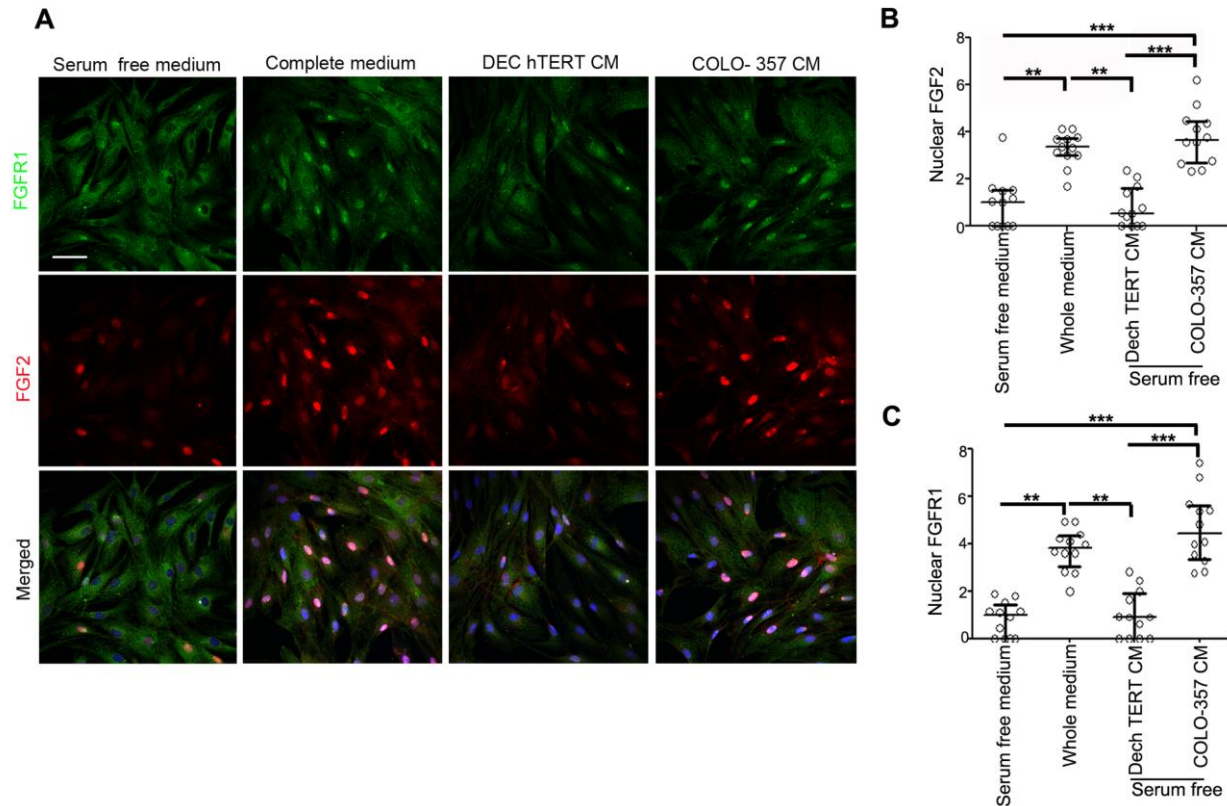


Figure 5.9 Nuclear FGFR1 and FGF2 translocation in stellate cells following treatment with cancer cell conditioned medium

(A) Shows an increase in nuclear FGFR1 (green) and FGF2 (red) following culture of PS1 cells with conditioned media (serum-free) from COLO-357 cancer cells and complete media (10% FBS) compared to conditioned media from Dec-hTERT normal epithelial cells or PS1 cells cultured in serum-free media (control). Quantified in B and C (normalised to PS1 cells cultured in serum-free media). Scale bar 20 μ m. ***P<0.001, ** P< 0.01. Kruskal-Wallis test with Dunn's post-test comparison. Data summary represented by median \pm interquartile range. Images are representative of at least three independent experiments.

5.2 Discussion.

5.2.1 *Three dimensional modelling reveals changes evoked in pancreatic cancer cells by stellate cells treated with PD 173074*

Using a 3D co-culture model of PDAC demonstrated the importance of the presence of stellate cells on pancreatic cancer cell invasion. In the absence of stellate cells, pancreatic cancer cells formed a thin layer on top of the ECM gel and failed to invade into the matrix. Thus, pancreatic stellate cells are required for cancer cells to invade. Indeed it appeared, upon immunostaining of gels with markers to distinguish cancer cells from stellate cells, stellate cells often preceded cancer cell invasion, suggesting that stellate cells form tracts in which cancer cells can follow. Indeed, this observation has also been observed by others in our laboratory, where it appears the extent of invasion is dependent on the ratio of stellate cell to cancer cells¹⁷¹.

Furthermore, it appears that cancer cells require direct cell-cell contact with fibroblasts, as previous studies have shown that, when stellate cells are embedded into the matrigel, cancer cells fail to invade¹⁶⁵. Indeed, co-culture of squamous cell carcinoma cells and cancer associated fibroblasts has demonstrated that, via Rho-mediated regulation of myosin light chain (MLC) activity and force-mediated matrix remodeling by fibroblasts, tumour cells move collectively within tracks laid down by the fibroblasts³⁷⁹. These results also highlight the importance of establishing a relevant 3D environment when generating organotypic models in order to establish a physiomimetic system. Our laboratory, as well as other groups generating 3D models that recapitulate various *in vivo* systems, have demonstrated this. Recently, Tian and colleagues have shown that FGF2 secreted by PSCs can

induce expression of the c isoform of FGFR1 in cancer cells when cultured in stellate cell conditioned media¹⁸⁶. This is of particular interest, as I have demonstrated that PS1 cells readily secrete FGF2 *in vitro*, and could represent one way in which stellate cells induce an invasive phenotype in pancreatic cancer cells in the organotypic model. Indeed, studies have shown that FGFR1 IIIc expression in spontaneously immortalised pancreatic ductal cells results in malignant transformation¹⁸⁸. Furthermore, FGF2 secreted by stellate cells is able to induce a down regulation of the FGFR1 IIIb isoform in cancer cells, which is associated with potent inhibition of the malignant phenotype³⁸⁰. This gives rise to an important paracrine signalling loop involving FGF2 derived from stellate cells that is able to induce the expression of FGFR1 IIIc in cancer cells. Furthermore, neutralising the effects of FGFs by heparin sepharose precipitation completely abolished these effects¹⁸⁶. This mechanism may contribute to the invasive phenotype of humanPDAC, which is based on tumour stroma interactions involving stellate cells. Furthermore, N-cadherin and FGF2 have been shown to act synergistically in breast cancer cells, to increase cell invasion and migration and to enhance the expression of matrix metalloproteinase 9³⁸¹. N cadherin prevents ligand mediated down regulation of FGFR1, leading to increased receptor signalling, sustained ERK activation³⁸² and could possibly provide a mechanism whereby FGF2 paracrine signalling from stellate cells may establish, and participate in, a more invasive pancreatic cancer cell phenotype.

Blocking FGFR signalling in our organotypic model resulted in striking effects on cancer cell and PSC invasion. Following treatment with PD 173074, there was a significant block of invasion of cancer cells into the ECM, and it was apparent that PSCs were 'trapped' within the overlying cell layer and exhibited a more rounded shape, distinct from the spindle myofibroblast like morphology shown by invading

stellate cells. This phenotype is indicative of a more quiescent stellate cell. Indeed, treating activated stellate cells with ATRA renders stellate cells quiescent¹⁷⁰, which has significant anti tumour effects on cancer cells, and cells change from a spindle shape to a more rounded form. It is yet to be determined if the stellate cells treated with PD 173074 are indeed rendered quiescent. However, results from cell cycle experiments suggest that stellate cells, following treatment with PD 173074, are not apoptotic, but are arrested in the G1 phase of the cell cycle. Studies by our group have shown that, for PDAC, the maximal effect on increasing cancer cell invasion as well as decreasing cancer cell apoptosis, occurs when stellate cells constitute the majority of the cellular population (maximal stellate cell proportion at 0.66-0.83) and accompanies changes in expression of key molecules such as E-cadherin and β catenin¹⁷¹.

An additional way by which signalling molecules can elicit distinct responses in different cell types is through activation or repression of other signalling pathways. This cross talk between signalling pathways results from specific interactions between signal transducing molecules, and the convergence or divergence of the programs of gene expression activated by each pathway. Combinations of signals may alter the effect of an individual pathway or one pathway may confer competence to a second signal. Recent results from our group suggest that pancreatic cancer cells show active Wnt signalling¹⁷⁰. Like FGF pathways, Wnt signalling is important in the embryological development of the pancreas and is reactivated or deregulated in PDAC³⁸³. Importantly, FGFs have been shown to interact with Wnts in a variety of developmental systems and in some cases the simultaneous activation of Wnt and FGF pathways leads to effects that are distinct from the individual effects of each factor³⁸⁴. A key event in the canonical Wnt pathway is the activation of β catenin, which subsequently regulates transcription of

specific target genes that modulate cell proliferation and apoptosis³⁸⁵. β catenin also functions at the cell membrane where, as a component of the adherens junction, it links cadherins to the cytoskeleton³⁸⁶. In certain contexts, studies have shown that β catenin can modulate the effects FGF signalling. For example, FGF2 treatment promotes translocation of β catenin to the nucleus and maintains the proliferation of multipotent neural stem cells³⁸⁷. Thus, FGF2 secreted by stellate cells in this coculture model may modulate the effect of Wnt signalling in cancer cells and fuel an increase in tumour cell growth. It would be of interest to assess the cross talk with Wnts and other known signalling pathways that interact with FGF signalling, such as TGF, IGF and Notch, that are known to be deregulated in pancreatic cancer cells⁵⁰. It is possible that targeting stellate cells by blocking nuclear FGFR1 and FGF2, which I have shown has a significant effect on stellate cell proliferation, may be sufficient to modulate the tumour microenvironment in such a way that is no longer permissive for cancer cell invasion. Interestingly, I did not see a significant effect on overall cell proliferation when organotypics were treated with PD 173074, however this is likely due to the dilution of the number in stellate cells in the organotypic cultures and the proliferation is representative of the cancer cell population, which is not effected by PD 173074 treatment.

Over the last decade, several therapeutics have been developed for the treatment of PDAC. While many show promise in simple 2D preclinical assays or xenograft mouse models, many fail when they are tested clinically including Gemcitabine, the current approved treatment for PDAC patients²⁸. This may be due to the oversight of the tumour microenvironment as a major factor impacting on cancer cell drug resistance, and many therapies have not been tested in a relevant system. Indeed, when I treated pancreatic cancer cells alone with PD 173074 in organotypic models or in 2D cell based assays, this had no effect on cells, confirming the importance of

the role of the tumour microenvironment and stellate cells on cancer cell behaviour. Importantly, the generation of genetically engineered mouse models (GEM) for PDAC that faithfully recapitulate the human disease, including resistance to Gemcitabine, has enabled new approaches to understand the importance of the tumour microenvironment in disease pathogenesis and therapeutic response. However, given the long latency and cost of the GEMMs, 3D organotypic models that mimic the morphological and functional features of their *in vivo* parental tissues are an attractive option for bridging the gap between cell-based discovery research and complex animal models^{388, 389}.

5.2.2 Nuclear FGFR and FGF2 mediate invasion of pancreatic stellate cells.

In vehicle-treated organotypic cultures, there was a significant increase in the number of PSCs with nuclear FGFR1 and FGF2 that were able to invade into the ECM. Following FGFR blockade, PSCs remaining in the cancer cell layer displayed mainly cytoplasmic staining for both FGFR1 and FGF2. These results suggest that nuclear FGFR1 and FGF2 may have a profound effect on PSC invasion, which in turn mediates cancer cell invasion.

Similar observations in isolated cancer associated primary stellate cells strengthens the idea that nuclear FGFR1 and FGF2 are important for stellate cell invasion and cancer cell behaviour, by not limiting studies to one stellate cell line. When I isolated and cultured primary stellate cells (after ethical permission was obtained), after a few passages the cells became quiescent, preventing their use in the original air-liquid interface model. However, the mini-organotypic model gave me robust comparable results to the original model. As more pancreatic tissue is becoming available, the expertise in isolating and culturing primary stellate cells, as well as

other stromal cell types (endothelial cells) to be used in co-culture with cancer cells in the laboratory, is increasing.

Despite the specificity of PD 173074 for FGFR1 inhibition, it can inhibit activity of other FGFRs as well as VEGFR2 activity²⁰². Given that VEGF is often secreted by cancer cells and can promote stellate cell activation¹⁵⁵, it was necessary to demonstrate the FGFR1 specific effects on stellate cell behaviour. Thus, the flexibility of the organotypic system allowed me to use PSCs that had been treated with FGFR1 RNAi to further confirm and demonstrate the specificity of FGFR1 on regulating stellate cell invasion and modulation of cancer cell behaviour.

Once in the nucleus, the role of FGFs in signalling and transcription has been extensively documented. In fact in some instances, the nuclear targeting of FGFs is required to elicit their full mitogenic response, which can be inhibited by mutation of the ligands NLS^{223, 390}. The nuclear roles of FGFs include regulating cell proliferation and DNA transcription and FGFs have been detected in rapidly proliferation tissues such as sights of injury and in cells of metastatic origin²²⁴. However, the functional role of nuclear FGFR1 is only beginning to emerge, despite the extensive literature on other RTKs, such as EGFR, being directed and convincingly located to the nucleus^{230, 234, 391}. However, the oncogenic significance of nuclear FGFR1 and FGF2 in an *in vivo* setting has not yet been established. Given the role of FGFR1 and other RTKS in driving proliferation, and by implication cell cycle progression, and the correlation with poor patient outcome, it not surprising that that the nuclear accumulation of this receptor and ligand could be oncogenic.

5.2.3 Signals from cancer cells can influence nuclear FGFR1 and FGF2

Culturing stellate cells in cancer cell conditioned media led to a significant increase in nuclear FGFR1 and FGF2. This is not surprising, as nuclear accumulation of endogenous FGFR1 is induced by changes in growth factors (such as FGF2, BMP7, EGF and angiotensin II³⁰⁴). PDAC cancer cells secrete a number of growth factors and cytokines that can activate PSCs, such as TGF β 1 and PDGF⁵⁸. How these factors may increase nuclear FGFR1 and FGF2 is yet to be examined. However, studies have shown TGF β can upregulate FGF2 expression via a TGFRII/Smad-3 dependent pathway³⁹². It is likely that other factors, not yet identified, are secreted by cancer cells and may induce nuclear activation. Nuclear FGFR1 can also be induced by changes in cell-cell contact and depolarisation³³¹, thus it may be possible that in a 3D co-culture environment, cancer-stromal interaction could also induce nuclear FGFR1 and FGF2 in PSCs. Further studies are required to investigate changes in PSC gene expression in appropriate physiologically relevant 3D culture conditions, in the presence of cancer cells, to define the exact role of paracrine and autocrine signalling.

Examination of PDAC sections revealed that a significant percentage of myofibroblasts at the invading tumour front had nuclear FGFR1 and FGF2, compared to myofibroblasts in the central core of the tumour. These results, coming from our 3D PDAC model, further emphasise the possible role of nuclear FGFR1 and FGF2 in driving PDAC tumour invasion. In PDAC, it appears the nuclear localisation of FGFR1 and FGF2 is critical in mediating the invasion of PSCs and, consequently, cancer cells. Although these results warrant further investigation, these data may provide a rationale for further targeting of this novel stromal pathway in clinical trials, in conjunction with conventional chemotherapy.

5.3 Conclusion

Taken together, I can conclude that the striking anti-invasive effect, seen when organotypic cultures were treated with PD 173074, is a consequence of a change in the cellular microenvironment provided by PSCs. *In vivo*, the stroma is appreciated as a major driver in promoting the aggressiveness of PDAC and makes up 80% of the tumour volume¹⁷⁰. The presence of PSCs in orthotopic models of PDAC increases distant spread of the tumour²¹⁷, with PSCs co-migrating with cancer cells to distant sites, likely aiding in the translocation of the tumour to the new microenvironment. It is possible that preventing nuclear FGF/FGFR mediated proliferation in PSCs leads to disruption of the tumour microenvironment, preventing pancreatic cancer cell invasion, thus identifying a novel therapeutic approach, targeting within the stroma of PDAC. A proposed model of possible mechanisms of nuclear FGFR1 and FGF2 in stellate cells is shown, based on the results found during my studies (Figure 5. 10). Furthermore, blocking nuclear FGFR1 and FGF2 in stellate cells, and the indirect effect this has on cancer cell behaviour, may have a wider impact than just in PDAC alone. The stellate system has been found in other organs such as the liver, kidney, intestine, spleen and lung¹¹⁹. Given that gene expression and functional studies have recently shown that hepatic stellate cells and PSCs share many homologies (expression of genes related to ECM proteins, contractility, retinoid metabolism and expression of growth factors)¹²⁶, it is possible that stellate cells from other organs may be targeted in a similar manner and this strategy may be extended to other disease types.

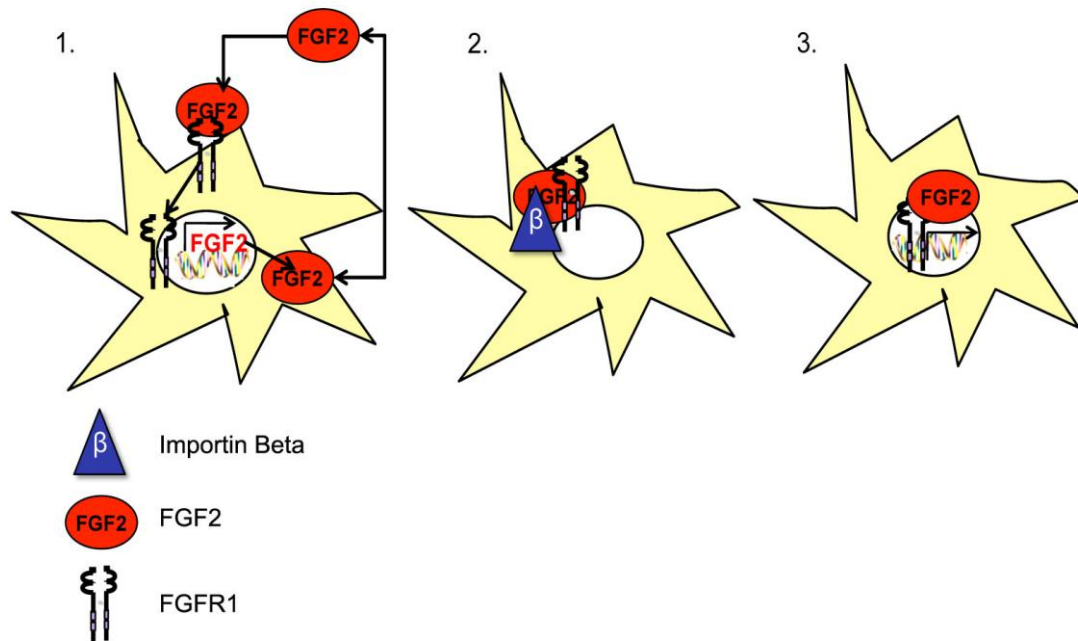


Figure 5. 10 Proposed possible models for the relationship between nuclear FGFR1 and FGF2 in stellate cells

Based on the results I have obtained from my studies I can hypothesise that there are three possible mechanisms for the relationship between nuclear FGFR1 and FGF2 in stellate cells. 1. FGF2, secreted by stellate cells, may bind and activate membrane bound FGFR1, resulting in receptor activation and internalisation. FGFR1 could then be translocated to the nucleus (possibly via endocytic pathway) where the receptor carries out its function regulating stellate cell proliferation by modulating *FGF2* gene expression. Newly synthesised FGF2 could be released and carry out its function in an autocrine loop, driving stellate cell proliferation. 2. HMW FGF2, which contains a NLS, may be required to chaperone FGFR1 to the nucleus, possibly in a complex with the nuclear import receptor, importin β . 3. FGF2 may associate with FGFR1 in the nucleus, possibly regulating receptor mobilisation and nuclear entry, in order for FGFR1 to carry out its function in regulating gene expression. In all three scenarios a relationship between nuclear FGFR1 and FGF2 exists, specifically driving proliferation in stellate cells.

5.4 Future challenges

3D cell culture models are valuable tools for studying the mechanisms of PDAC, providing an easily manipulated system in which specific questions can be addressed, thus facilitating the translation of basic science to clinic. Although no single *in vitro* model could entirely replicate the ecosystem of PDAC, or provide a complete understanding of the fundamental mechanisms governing pathogenesis, organotypic models have advanced our understanding of the molecular and cellular mechanisms of PDAC. By providing an environment in which cell behaviour and novel treatment options can be investigated in an easily reproducible and controlled manner, these models more precisely mimic PDAC, thus providing a major contribution to preclinical drug and therapeutic discovery. A huge advantage of the organotypic system is that any component of the model can be readily modulated in a short time frame. For example, the matrix composition can be altered to reflect the *in vivo* situation. The increase in ECM stiffness exerts elevated force on transformed cells increasing cellular response and resulting in increased tumour growth, survival and motility^{171, 393}. Additional cell types, such as or endothelial cells, can be titrated in. This is being tested in our laboratory by Dr Francesco DiMaggio and shows promising results. In oesophageal cancer, to assess the role of stroma on angiogenesis, endothelial cells on a 2D monolayer have been cultured with fibroblast and cancer cells embedded in a collagen gel layered on top³⁹⁴. Another example includes investigation of the role of macrophages in malignant growth of human squamous cell carcinoma³⁹⁵. Immune response and inflammation play an important role in the desmoplastic reaction and inflammation is thought to activate pancreatic stellate cells^{139, 396}. Thus this model would be particularly pertinent to PDAC.

In addition pancreatic tumours are characteristically hypoxic. This hypoxia is exasperated because of hypovascularity with profuse stroma which provides an environment in which PCCs thrive.³⁹⁷ Hypoxia is a driving force in PDAC progression, contributing to resistance to chemo- and radiotherapy as well as increasing tumour metastasis and contributing to poor patient survival^{398, 399}. It has been shown that under hypoxic conditions (1% oxygen)⁴⁰⁰, PSCs may influence PCC invasion more strongly than those in normoxia, thus it may be more reflective of the *in vivo* situation to culture organotypics in a hypoxic environment and treat with PD 173074 to assess the effect on cancer cell invasion.

Finally, many PDAC patients present very late with their disease when metastases have already occurred. Thus, treating PDAC cells in a 3D environment when they are first assembled does not reflect the true clinical setting as tumours are already established at the time of patient treatment. Currently Miss Elisabeta Carapuca is investigating the effect of treatment of organotypic models once they are established and invasion of PDAC and/or stromal cells has begun. It is likely this would give a better understanding of the treatment regimen that is required when new novel therapies emerge into a preclinical setting. It would be particularly important to assess the effect of blocking nuclear FGFR1 and FGF2 in stellate cells once the organotypics are established and assess the effect on cancer cell invasion. In addition it would be interesting to assess any potential benefit of blocking nuclear FGFR1 and FGF2 in PSCs using PD 173074, in combination with standard cytotoxic agents and assess the effects of cancer stroma cross talk using the organotypic model. Preclinical studies have shown that histone deacetylase (HDAC) inhibitors may down regulate FGF2 expression⁴⁰¹, thus it would be of interest to assess if treating 3D organotypics with PD 173074 and an HDAC inhibitor would have

synergistic effects in modulating the tumour microenvironment and offer a new treatment modality in PDAC.

CHAPTER VI CONCLUDING REMARKS

FGF signalling has been heavily implicated in the molecular pathology of pancreatic cancer. PSCs have been identified as the key cells responsible for the stromal reaction in PDAC and chronic pancreatitis and have been identified as a major contributor to aberrant FGF signalling in PDAC^{121, 402}. Thus targeting FGF signalling in PDAC offers an attractive target. The availability of the hTERT immortalised pancreatic stellate cell, PS1 has enabled me to investigate the effects nuclear FGFR1 and FGF2 in an organotypic model that recapitulates the PDAC situation in a physiologically relevant manner. Other groups have used immortalised PSCs, however these lines did not express key stellate cell markers including desmin and GFAP^{174, 403}. Moreover, the use of SV 40 to immortalise these cells can induce genetic changes and could possibly generate a cell line that does not correctly represent characteristics of 'normal' stellate cells (non tumour associated)⁴⁰⁴. The PSC line in our laboratory (PS1) is sourced from human pancreas and immortalised using hTERT and, after more than 5 years of continuous culturing, these cells still express stellate cell markers including desmin, GFAP, vimentin and α SMA¹⁷⁰.

The strength of my experiments lies with use of a range of pancreatic cancer cell lines, ranging from well to poorly differentiated, as well as primary stellate cells (cancer associated) isolated from different patients, as well as PS1 (taken from normal pancreas), to thoroughly scrutinise the role of nuclear FGFR1 and FGF2. I have shown that these cancer associated stellate cells demonstrate the true characteristics of PSCs. Studies by Apte and colleagues have shown that stellate cells isolated from normal pancreas and PDAC tissue can stimulate tumour growth similarly *in vivo*, suggesting that 'normal' pancreatic stellate cells are rapidly

activated when exposed to cancer cells¹⁷⁵. When I isolated primary pancreatic stellate cells, I was only able to passage the cells for a limited number of times before the cells became senescent. It would have been ideal to use primary stellate cells isolated from different patients in all of my models to investigate the effect of nuclear FGFR1 and FGF2 on cell behaviour, however the expertise in our laboratory at culturing these cells is increasing and it may be possible to generate sufficient quantities of primary PSCs in the future. Nevertheless, given the similar results of nuclear FGFR1 and FGF2 obtained from using the primary isolated stellate cells and PS1 cell line, both 2D cell culture assays and in the modified 3D mini organotypic co-culture model, the relevance of the results using the PS1 cell line can be confidently interpreted. Furthermore, the effects of nuclear FGFR1 and FGF2 observed *in vitro* on the behaviour of stellate cells could be recapitulated in human PDAC tissue. Examination of PDAC sections revealed that a significant percentage of myofibroblasts at the invading tumour front had nuclear FGFR1 and FGF2, compared to myofibroblasts in the central core of the tumour. This further emphasises the possible role of nuclear FGFR1 and FGF2 in driving PDAC tumour invasion.

Developing new therapeutics has been a challenge for PDAC¹³¹. PDAC still carries one of the bleakest prognoses in all of medicine and there is an urgent need for therapies. One of the possible reasons that targeted therapies fail to improve the prognosis of patients with PDAC may, in part, be explained by the diverse influences exerted by the tumour microenvironment. Delineating the signalling networks within the tumour microenvironment, may help to explain the huge discrepancy between relative success and effectiveness of therapies in preclinical assay (predominately 2D cell based assays and xenograft mouse models) and their abject failure in human PDAC, in which the stroma is not well represented. Furthermore, PDACs

harbour a plethora of heterogeneous genetic abnormalities. Performing a comprehensive genetic analysis of 24 cancers, Jones and colleagues showed that PDACs contain on average 63 genetic alterations, the majority of which are point mutations. These alterations defined a core set of 12 cellular signalling pathways and processes that were genetically altered in 67-100% of the tumours. However, the pathway components that are altered in any individual tumour differ greatly and would thus entail combinations of agents such as chemotherapy and targeted therapy that broadly target downstream mediators or key pathways⁷³. Until a more tailored approach is feasible to treat PDAC on an individual basis, targeting the stroma is an attractive approach. I have shown that using an FGFR inhibitor can specifically target nuclear FGFR1 and FGF2 in stellate cells and ultimately modulate the tumour environment into one that is not permissive for cancer cell invasion. The advantage of this system is a possible prevention of the escape or adaptation of cancer cells, which may lead to tumour resistance.

Although the organotypic model provides a physiologically relevant means to study the tumour stroma interactions and the use of new therapies to target this cross talk, it still remains a simplified representation of the complex *in vivo* situation. However, the use of the organotypic model as a preclinical tool is becoming increasingly important and our laboratory, as well as others, are modulating the 3D cultures to recapture other important aspects of the tumour microenvironment that can influence cancer cell behaviour including vascularity and inflammation. Thus, 3D organotypic models have potential for bridging the gap between cell-based discovery research and complex animal models^[388, 389]. The next step will be to test the therapeutic potential of blocking nuclear FGFR1 and FGF2 in stellate cells by treating a mouse model with an FGFR inhibitor, or an FGFR inhibitor combined with the current standard of care, Gemcitabine. One appropriate model is the KPC

mouse which express mutant *KRAS* and *TP53* alleles in pancreatic cells and develop tumours that strongly recapitulate human PDAC, including a strong desmoplastic reaction¹¹⁴. This is likely to give a reliable prediction of the effect of FGFR inhibitors as a new therapy for patients with PDAC.

Several pharmaceutical companies have developed FGFR tyrosine kinase inhibitors that are in early phases of clinical trials^{265, 405}, however preclinical development of potent FGFR TK inhibitors has been complicated by some side effects. For example, FGF23 is involved in phosphate homeostasis and, in preclinical models, highly potent FGFR tyrosine kinase inhibitors have caused hyperphosphatemia-mediated tissue calcification by blocking FGF23 signalling¹⁴. It is not known if this will be a problem in human trials. Thus, it is even more critical that the targets with which nuclear FGFR1 and FGF2 may specifically regulate stellate cell behaviour are identified in order to develop new targeting strategies and reduce toxicity.

Finally I propose that preventing nuclear FGF/FGFR mediated proliferation in PSCs leads to disruption of the tumour microenvironment, preventing pancreatic cancer cell invasion, thus identifying a novel therapeutic approach targeting within the stroma of PDAC and may lead to a better outlook for patients with this deadly disease.

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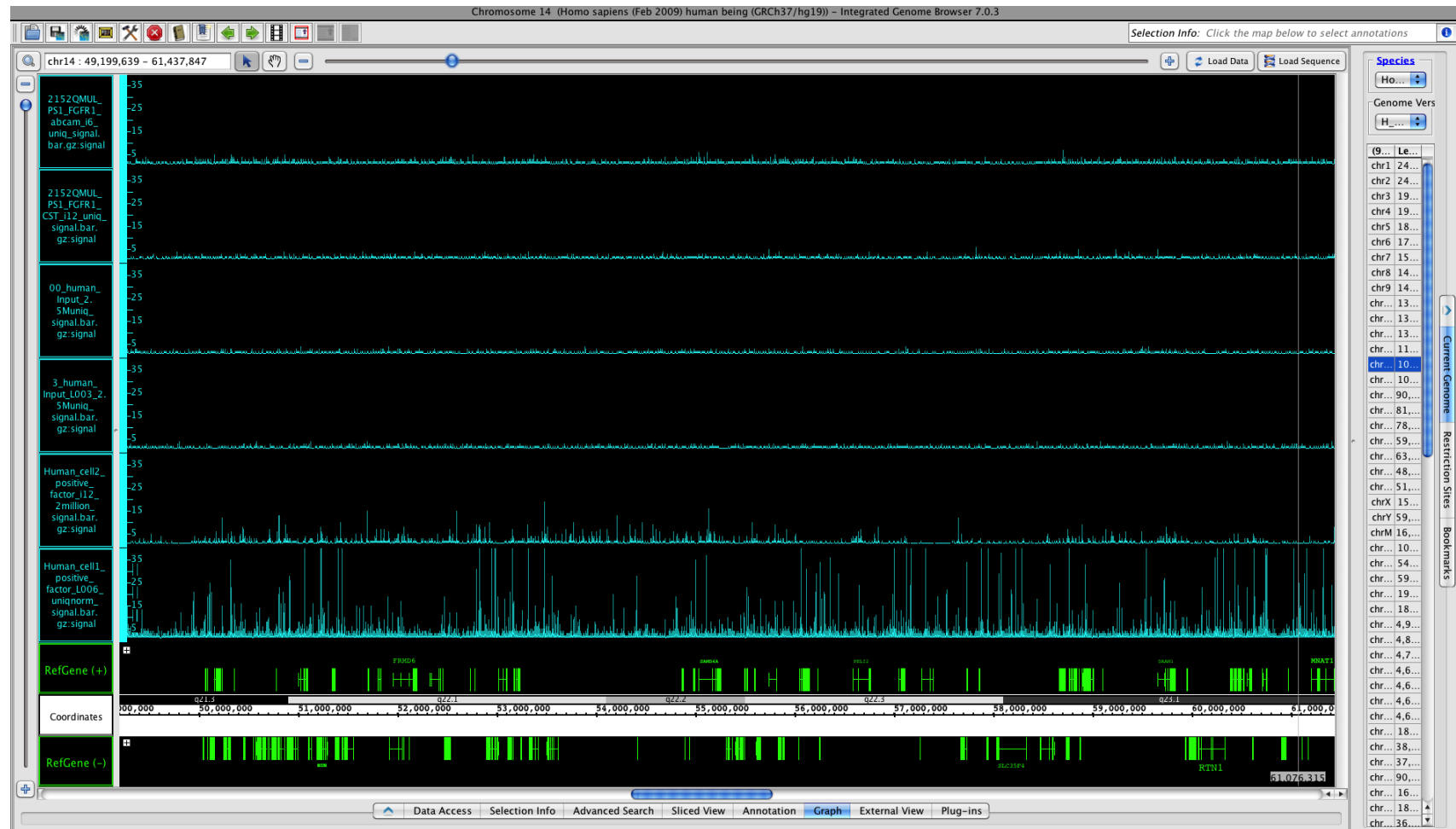
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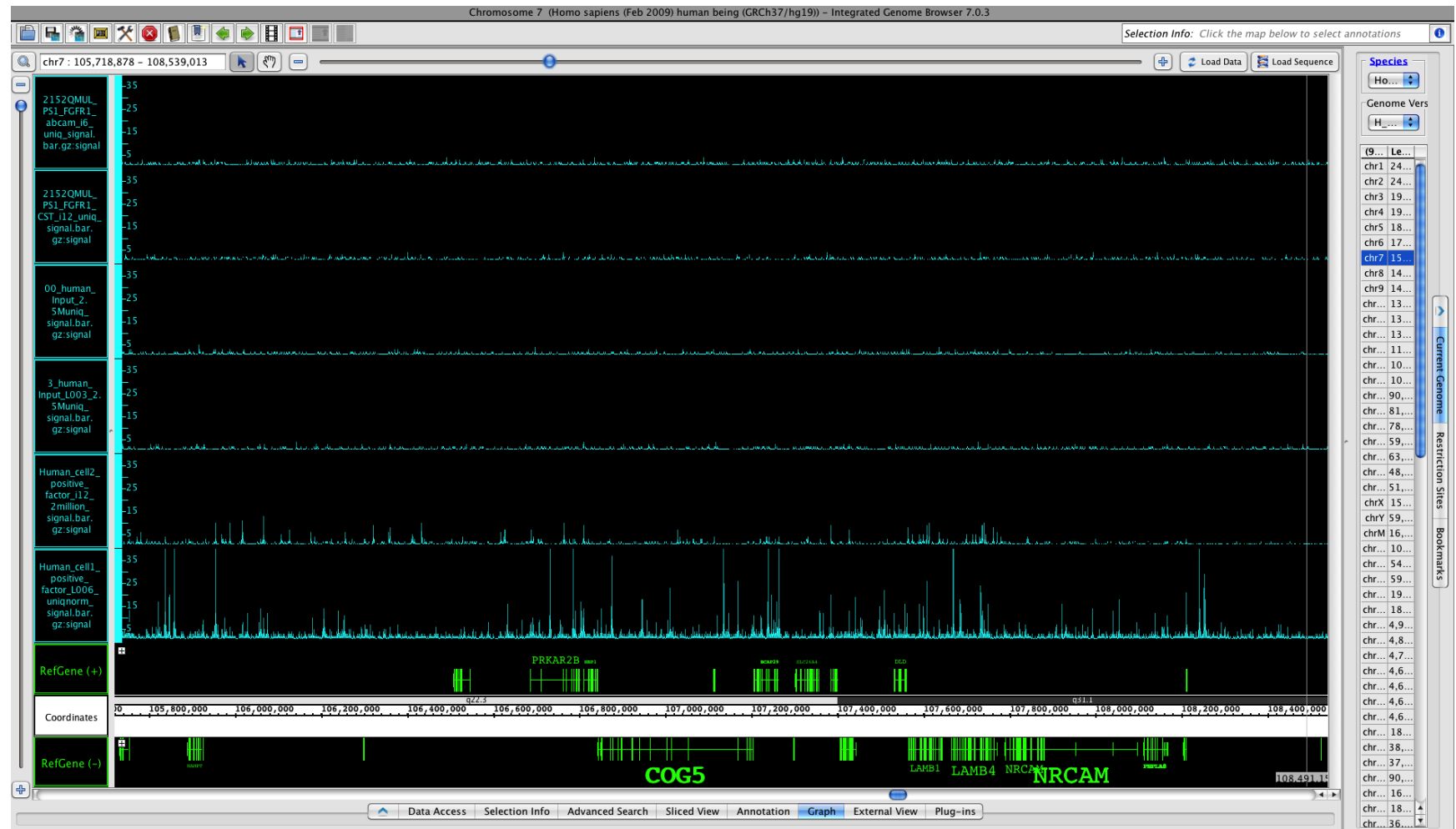
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Appendix

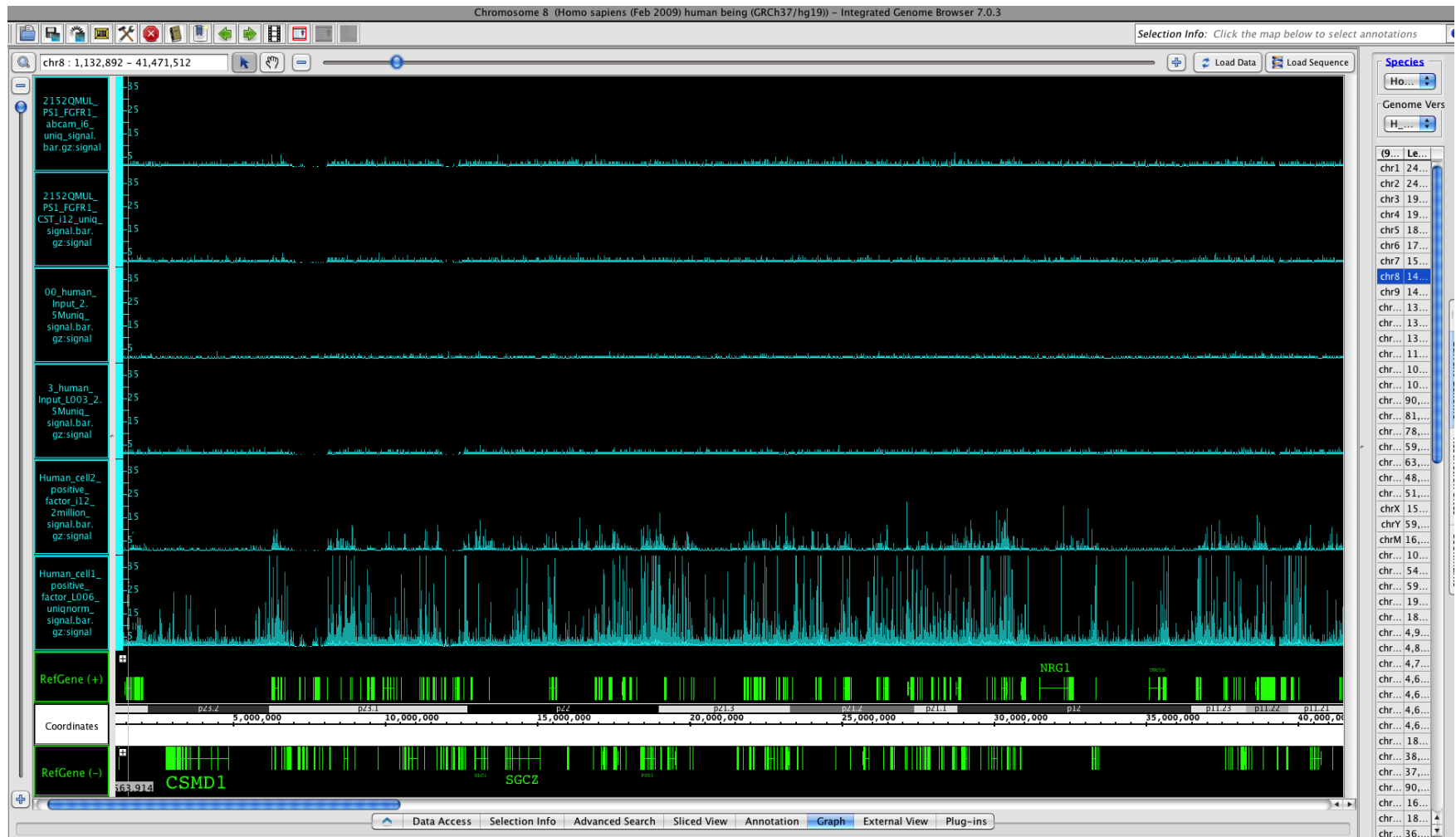
A



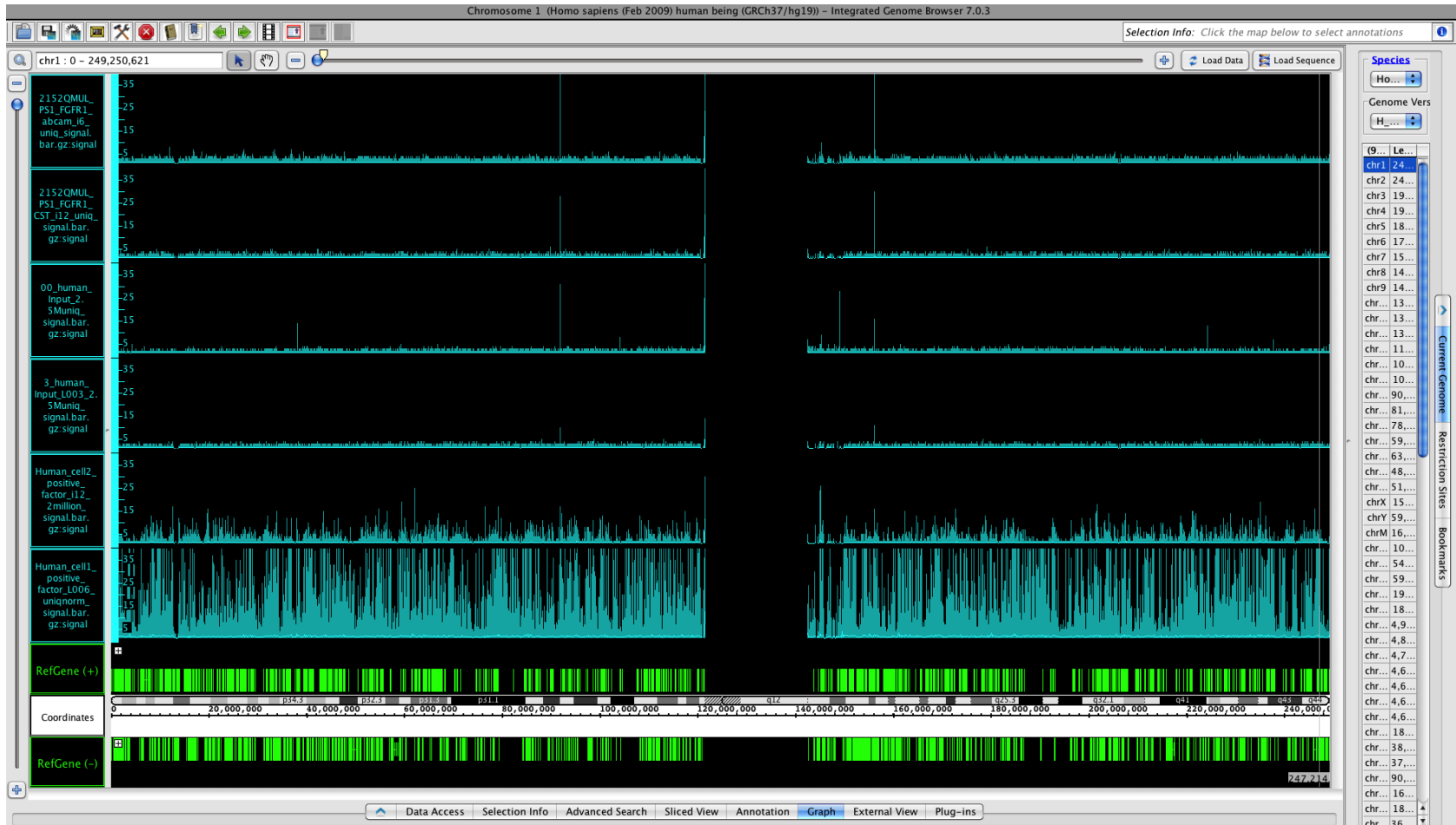
B



C



D



Two ChIP reactions were carried out using 30 µg of PS1 cell chromatin and anti-FGFR1 antibodies from either Abcam (ab10646) or Cell Signaling (9740). The ChIP DNA was processed into a standard Illumina ChIP-Seq library and MiSeq sequenced to generate >2 million reads. Approximately 5 million reads per sample were aligned to the human genome (hg19 assembly) and, after removal of duplicate reads, 2.4 and 1.4 million alignments were obtained for the Abcam and Cell Signaling immunoprecipitated samples, respectively. A signal map showing fragment densities along the genome was generated and visualised in the Integrated Genome Browser (IGB). In addition, MACS peak finding was performed to identify the most significant peaks. Using default settings (without control file), 189 and 127 peaks were identified.

These peak numbers are essentially at the background level, as 100-250 peaks are typically also obtained with Input DNA. Images show the four IGB screenshots of different chromosomes (A) Chr 14, (B) Chr 7, (C) Chr 8, (D) Chr 1. Each one shows the FGFR1 data in the top 2 tracks. The 3rd to 6th data tracks of the screenshots (turquoise color) show 2 negative and 2 positive controls for comparison. These controls were also generated with 2.0-2.5 million tags. Two controls each are shown to illustrate the variation in background noise seen in the negative control (tracks 3 and 4), and the range of peak signals seen in successful antibody test validation assays (tracks 5 and 6). The 2 tracks at the very bottom with the green boxes show the RefSeq genes in the plus and minus strand. The results show that the FGFR1 ChIP-Seq data look almost identical to the Input control (negative control), thus there are no significant peaks. In conclusion, the antibodies did not identify significant binding between FGFR1 and any specific regions of the genome in ChIP Seq. This may be due to lack of antibody specificity for ChIP, or may reflect an absence of FGFR1 binding. These possibilities are now the subject of further investigation in the lab